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(71) Applicant (for all designated States except US): F.B. INVEST-MENTS PTY. LTD. [AU/AU]; 825 Stanley Street, Wool-loongabba, QLD 4102 (AU).

(72) Inventor; and
(75) Inventor/Applicant (for US only): TEASDALE, Robert, Dixon [AU/AU]; 33 Maculata Drive, Chapel Hill, QLD 4069 (AU).

(74) Agent: PIZZEYS PATENT AND TRADE MARK ATTOR-NEYS; Level 6, 444 Queen Street, Brisbane, QLD 4000 (AU).

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(54) Title: A PLANT AND METHOD OF MODIFICATION

(57) Abstract

A method of modifying a plant to increase vegetative growth of commercially valuable plant structures at the expense of non–essential and non–commercial structures is provided. From reproductive tissues of both *Pinus radiata* and *Eucalyptus grandis*, cDNA were isolated corresponding to genes which were specifically expressed during early development in both male and female plant structures. The promoter regions to these genes were isolated from respective genomic DNAs and fused to a structural gene for a bacterial ribonuclease, Barnase. In a variation of the method, an expression control system was used to control promoter leakage. Gene constructs were also prepared which expressed anti–sense forms of essential MADS box genes for these plants. Candidate gene constructs were used to transform shoot–forming tissues of target eucalypt and pine species. Explants from successful transformants were assessed for incorporation of the gene constructs and production of sterile plants.

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A PLANT AND METHOD OF MODIFICATION

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of Application Ser. No.08/717971, a continuation of Ser. No.08/384208 (ABANDONED) filed 3 February, 1995, which was a continuation-in-
5   part of Application Ser. No.08/210730 (ABANDONED) filed 18 March, 1994, which was a continuation-in-part of Application Ser. No.08/039419 (ABANDONED) filed 15 April, 1993, being the United States National Phase of PCT/AU91/00445 filed 26 September, 1991.

10   ### FIELD OF THE INVENTION

This invention relates to new plants and methods of modification thereof.

This invention has particular but not exclusive application to forestry and increasing the productive capacity of trees, and
15   for illustrative purposes reference will be made to such application. However, it is to be understood that this invention could be used in other applications, such as the modification of other plants such as certain leafy food crops to increase the production of useful parts thereof.

20   ### BACKGROUND OF THE INVENTION

It is well recognized that the useful structures of domestic and commercial plants are in competition in the plant with other non-essential structures for growth resources such as water, nutrients and photosynthesis products. For example, the
25   development of reproductive structures on forest trees represents a significant burden on the resources of the trees, the reproductive effort occurring at the expense of vegetative growth. The extent of the effect of reproductive burden on vegetative growth may be estimated by measuring the proportion
30   of photo-assimilate which is directed to flowers and fruits, or strobili and cone growth for gymnosperms. In conifers,

reproductive effort is variously estimated to be up to 16% of annual photosynthate.   The formation of reproductive structures imposes a burden on the tree beyond that of carbon allocation. First, these structures have very large requirements for

5   important nutrients at the expense of vegetative tissues so that, at least under conditions of nutrient deficiency, prevention of reproductive structure formations as proposed can lead to a greater increment in vegetative growth than the corresponding mass of reproductive structure otherwise formed.

10   Nutrient losses from pollen dispersal are not inconsiderable in most forest plantations, with an estimated annual production of 370 kg/ha of nutrient-rich pollen by Pinus radiata plantations. Tree crop productivity of P. radiata is as yet only about 50% of that theoretically obtainable on the basis of

15   incident solar radiation. Therefore, in many environments, there are good prospects for significant increase in harvest yield from trees which have been inhibited from producing reproductive structures.

          It is noted that there is a deleterious effect in at least

20   P. radiata on the value of timber caused by persistent stem cones, and research has been advocated for methods for chemically inducing cone abscission in species such as P. radiata. However, it is clear that means involving chemical treatment of forest trees will involve considerable expense in manufacture and

25   application, this cost being exacerbated in certain circumstances where environmental factors both preclude indiscriminate broadcast of such chemicals and insist on strict control of run off.

          Seeds from mature trees will produce an undergrowth of

30   saplings which must be removed periodically to prevent competition with the tree crop for soil resources and to minimize

the risk of damaging fire.   This sapling removal is labour
intensive, time consuming and accordingly expensive, adding
considerably to the production cost of farmed forest timber.
Trees and other plants may form reproductive structures at their
5   apical meristem, leading to unwanted termination of vegetative
growth at this apice.   Such terminal flowering may effect the
form of trees, with heavier branching which reduce the quantity
and value of harvestable timber if the trees did not continue to
grow vegetatively.

10   US Patent No. 5122466 discloses the transformation of
Loblolly pine by bombardment with microprojectiles carrying an
expression cassette to yield transformants.   The disclosure
provides a method establishing that a plasmid bearing a
functional gene construct can successfully transform plant cells.
15   However, the disclosure does not provide for inducing sterility
in plants.

Koltunow et al. (The Plant Cell, Vol2, 1201-1224) discloses
methods of inducing male sterility in plants by expression of a
lethal gene (Barnase) in plant stamen cells (tapetal).   A
20   chimeric gene comprising the Barnase gene inserted into and under
the expression control of the TA29 gene of tobacco, a gene
established to be specifically expressed in tapetal tissue.   The
disclosure relates to the induction of sterility only, but does
not overcome the problem of plants developing reproductive
25   structures at the expense of vegetative growth.

The present invention aims to alleviate the above
disadvantages and to provide plants of enhanced productive
capacity and methods for the production thereof which will be
reliable and efficient in use.   Other objects and advantages of
30   this invention will hereinafter become apparent.

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SUMMARY OF THE INVENTION

With the foregoing and other objects in view, this invention in one aspect resides broadly in a method of enhancing vegetative growth in a plant including the steps of:-

5   identifying a gene having a substantially tissue-specific promoter expressing during the development of both male and female plant reproductive structures;

constructing an expression cassette comprising a heterologous coding region capable of expressing a product which

10   aborts said development under the expression control of said promoter;

transforming plant cells with said expression cassette, and selecting and vegetatively propagating the transformants.

Preferably, the non essential structure is selected from the

15   reproductive structures of plants which are economically capable of artificial vegetative propagation. Preferably, the plant selected is a tree for timber, pulp or fibre production wherein poor or absent expression of reproductive structures may result in increase in vegetative growth of the valuable material.

20   A large number of genes are differentially expressed between sexual and vegetative buds. Accordingly, it is preferred that the gene be selected from those specific to sexual budding, specific to the production of other sexual structures, or specifically coding for a product essential in a developmental

25   pathway for a reproductive structure. The gene is preferably selected for its early expression, specifically in the developing reproductive tissues.

For example, five cone-specific genes displaying strong homology to *Arabidopsis thaliana* and *Anthirrhinum majus* floral

30   meristem and organ identity genes have been identified in *P.radiata* cDNA library prepared from immature female and male

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cones.   Three of them, PrMADS1, 2 and 3, belong to the family of

MADS-box genes showing homology to *Arabidopsis AGL-2, AGL-4* and

AGL6 genes and *dall* gene from another non-angiosperm, *Picea

abies* (Norway spruce), respectively.   The PrFL1 gene is the pine

5   ortholog of *Arabidopsis Leafy (Lfy)* and *Floricaula (Flo)* gene

from *Anthirrhinum*.   The *PrCon1* shows strong homology to

Arabidopsis CONSTANS (CO) gene.   A significantly lower level of

expression was detected in vegetative tissues: vegetative buds,

needles, stem and roots.   *In situ* hybridisation showed that

10   expression of these genes is substantially detectable only in

reproductive tissue cells.

Expression analysis has revealed that all five genes show

different patterns of expression in different stages of

development of male and female cones.   PrMADS1, 2 and 3 genes are

15   cone-specific in that expression of both genes was substantially

restricted to reproductive organ primordium tissues.   No

detectable expression of these genes was observed in vegetative

tissues such as vegetative buds, needles, stems, roots.   For

PrFL1 and PrCon1 low detectable expression was observed in

20   vegetative buds.

In reproductive organs low level of expression of both genes

was detected at early stages of cone development (5 mg cones)

which was increased during a cones development (50 mg cones).

In male cones expression of both genes was restricted to

25   microsporangium containing primary sporogenous cells.   In female

cones expression of both genes was restricted to premature

ovules.

In a further example, MADS-box genes of *Eucalyptus spp.* have

been identified as having the reproductive organ specificity

30   required of the present invention and identified hereinafter as

genes EGM1, 2 and 3 respectively, gene EGM3 being highly specific

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for reproductive primordia.

Preferably, the gene is identified in cDNA libraries prepared from mRNA isolated from reproductive tissues and selected by differential screening against mRNA of vegetative structures. Since the biochemical pathways for vegetative buds and developing reproductive structures may include common expression products and consequently similar mRNAs, it is preferred to differentially screen cDNA libraries against vegetative bud mRNA, with or without preceding enrichment of the cDNA for genes specifically expressed in reproductive tissues. The selected gene may be any which when its expression is blocked or otherwise made ineffectual, results in the failure to produce a non essential plant structure.

Alternatively, the cDNAs may be utilized as probes to select corresponding genomic clones from genomic libraries. The genomic clones may be used to isolate and identify gene promoters that specifically express genes unique to reproductive structures. Such promoters can then be combined with lethal genes which when expressed will inhibit or terminate growth of the cells within which the lethal gene is expressed. The promoter-gene fusions when stably incorporated into a plant, by any suitable known means, will result in failure to develop reproductive tissues in whole or in part.

Candidate cDNA clones may be raised and are preferably selected for the presence of genes expressed in both male and female buds. The selection may be directed to those genes which are naturally produced or those which are induced by plant growth regulators which promote flowering, such as various gibberellins. Preferably, the specific expression in floral tissues is confirmed using an in situ RNA hybridization procedure with a wide range of different plant tissues.

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The preferred cDNA clones are those further selected for the characteristics of early appearance and highly specific expression.

The modification of the identified gene may be by fusing the tissue-specific promoter of said gene with a structural gene for a deleterious or lethal product such that regenerated plants transformed with said gene-fusion will not form said non-essential structure. Alternatively, a critical function of said gene may be disrupted or modified by expression of the modified gene in transformed plants.

The modified gene is preferably introduced into a plant normally containing the identified gene such that a critical function of the identified gene is disrupted or modified. However, it is also envisaged that introduction of the modified gene into plants not containing the specifically identified gene may result in useful reduction or elimination of a non-essential plant structure, particularly where the gene selected has analogues represented across several species, or in closely related species where the corresponding gene is essentially homologous with the gene in question.

Modification of the gene may be achieved by any suitable means, with the expression strategy desired being the primary arbiter of the modification process utilized. For example, it may be intended to constitutively express an antisense or perhaps ribozyme version of a gene which is critical to development of reproductive structures, so that the normal gene action is disrupted and vegetative development occurs instead. The method may be to splice a promoter specific to a reproductive structure with a lethal gene which codes for an expression product which will cause abortion of the tissue in which it is produced, for example, buds that differentiate as reproductive structures.

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Genes expressing antisense RNA against the mRNA coded by each of the selected target genes may be constructed. These and shorter RNA sequences that bind to the initiation regions of the target mRNA may be used for inhibition of translation. Of

5   course, other critical regions apart from the translation initiation site may be targeted for binding of antisense RNA.

One option is to use an antisense or ribozyme version of a critical house-keeping gene, such as the actin gene or a gene coding for an enzyme of aromatic amino acid biosynthesis, for

10 example enolpyruvyl shikimate phosphate synthase. Alternatively, a deleterious enzyme such as a protease, ribonuclease, or deoxyribonuclease may be encoded for biosynthesis under control of a sexual promoter or any promoter specifically expressed in a non essential structure.

15 One difficulty with the tissue ablation approach is the effect of promoter leakage or expression in other tissues. Accordingly, where the promoter controlling the heterologous coding region in expression is merely substantially tissue specific, any residual leakage is preferably overcome by

20 modification of the expression cassette to include appropriate leakage control. For example, to overcome leakage a second copy of the substantially tissue specific promoter, or a second tissue specific promoter, may be used to promote a gene producing a control product capable of switching on production of an

25 inhibitor to the lethal gene product in non target tissues. One example of this approach is to construct an expression cassette including a control system (gene cascade) whereby expression of a lethal gene(s) is countered in non-target tissues, such as cassettes carrying both Barnase and Barstar where Barstar is

30 under the constitutive control of a promoter responsive to repressor *lacIq*, itself promoted by a second copy of the selected

tissue specific promoter.

In the case of *Eucalyptus* transformation and regeneration, there are particular features of the species which have required the development of specific transformation protocols. Accordingly, in a further aspect this invention relates to a novel method for *Agrobacterium*-mediated transformation of *Eucalyptus* shoot and seedling explant tissue and regeneration of plants. *Eucalyptus* species are known for their fast growth and high biomass productivity. They are grown in commercial forestry plantations around the world. Reforestation, the controlled regeneration of forests, has become an integral part of forest management in order to secure a renewable and sustainable source of raw material for production of paper and other wood-related products. Forest trees can be regenerated by either sexual or asexual propagation. Sexual reproduction of seedlings for reforestation has traditionally been the most important means of propagation. Regeneration by seed usually results in highly variable progeny. On the other hand, vegetatively propagated clonal planting stock have a number of potential benefits including uniformity in quality and growth.

Eucalypts are primarily native trees to Australia with a small number of exceptions in the Asian region. However, they are now widely grown around the world. Several species of *Eucalyptus* are fast growing trees of importance for timber and paper pulp industries. Genetic improvement of eucalypts, as of other trees, is difficult with traditional breeding because of the constraint imposed by long developmental cycles.

During the last two decades, substantial progress has been made in genetic engineering of plants. However, progress in the application of biotechnology to trees has been slow and there have been only a few reports concerning *Eucalyptus* (Teulieres *et*

al., 1994). Assessment of these reports is difficult as the technical details of these reports are generally not available. However, these studies have concentrated on the good regeneration properties of *E. camaldulensis* seedling explant tissue. The main

5   difficulties for Eucalyptus transformation appeared to be in establishment of suitable regeneration systems as well as the low transformation frequencies. Biolistic bombardment has been tested as an alternative to *Agrobacterium*-mediated transformation, although transient expression was observed (Rocharge *et al.*,

10   1995), transformed *Eucalyptus* plants were not regenerated. Extensive work has been performed on transformation through electroporation of protoplasts (Teulieres *et al.*, 1994). However, there has been only one report concerning successful transformation of *Eucalyptus* protoplasts (Kawasu *et al*, 1991).

15   There are no reports on the transformation and regeneration of *Eucalyptus* shoot material. Shoot explants are generally used for the micropropagation of elite clones.

There is an urgent need to increase the quantity and quality of fast growing timbers in the future and the micropropagation

20   of elite transgenic *Eucalyptus* can play a significant role.

It is therefore, desirable to provide a method for the introduction of genes into *Eucalyptus* species for mass production of clones of genetically modified and improved trees. It is further desirable to provide a multistage regeneration protocol

25   which can be utilised effectively on *Eucalyptus* species to produce large quantities of plants for field planting. It is further desirable to provide a progression of steps which, in combination, provide a method for transformation and regeneration of diverse genotypes and species of *Eucalyptus* and *Eucalyptus*

30   interspecies hybrids. In addition, it is desirable to combine gains from conventional genetic testing and breeding with

molecular genetic improvement to produce a high value-added
Eucalyptus plant.

These objects and others are addressed by the present
invention, which is designed to produce elite Eucalyptus plants.
These objectives are achieved by a multi-step method for the
regeneration of Eucalyptus plants. Although other transformation
and regeneration protocols have been published, none of these
methods have proven totally effective with the Eucalyptus species
in that none enabled the practitioner to reliably proceed from
the beginning step of shoot explant transformation to completion
of the regeneration process resulting in establishment of plants
in field conditions. The present protocols provide such a multi-
step method for Eucalyptus plants. It incorporates two key steps.
The first is keeping the shoot explant intact and upright during
the co-cultivation step with Agrobacterium so that the leaves do
not touch the tissue culture medium. The second key step is the
incorporation of a liquid culture selection stage. This liquid
culture selection step is only effective once regenerated shoots
have been produced on solid medium. Shoot material grew faster
in liquid medium and formed more shoots when compared to shoots
grown on solid medium. This liquid selection step has the
advantages of reducing false positives by increasing selection
pressure, reducing residual Agrobacterium and increasing the
amount of shoot tissue compared to solid grown cultures.

Typically, the process may proceed according to the
following steps:

1. The Agrobacterium-mediated transformation of Eucalyptus
explant material with DNA. The source of the explant material may
be from seedlings or micropropagated clonal plant tissue.

2. The Agrobacterium and explant are co-cultivated. In
the case of shoot explants, the shoots are kept upright in the

solidified medium so that the leaves are not in physical contact with the medium.

3. The explants are then transferred to medium containing an antibiotic which kills the *Agrobacterium*. This selection is maintained for the remainder of the plant regeneration procedure.

4. The explants are then transferred to callus induction medium containing a selective agent which only allows the growth of tissue containing the introduced DNA. This selection is also maintained for the remainder of the plant regeneration procedure.

5. The explants are then transferred to shoot induction medium.

6. The regenerated shoots are then put into in liquid culture.

7. Surviving shoots and callus are transferred to solid medium.

8. Putative transformed shoots are assayed for presence of the introduced DNA.

9. Transgenic shoot material is then transferred to root induction medium.

10. After root elongation and development, the plantlets are transferred to soil or other growing medium in a container where root development and acclimatisation (gradually lowering relative humidity) continue.

12. Clones can be micropropagated by tissue culture propagation techniques and grown into trees of a size and form suitable for planting.

There are several advantages inherent with the use of this novel method. For example, the method is well suited for large-scale production of clones of genetically modified and

improved *Eucalyptus*.   The present processes also provides a reliable multi-step regeneration method for the recalcitrant *Eucalyptus* species. It is the combined application of the progression of steps in this novel multi-step method that has

5   enabled the first successful field planting of many different genotypes of *Eucalyptus*.

Apart from the use of infective agents such as *Agrobacterium* for transformation of pines and eucalypts, it has now been determined that new protocols for transformation

10   by biolistic bombardment are useful for pines.   Transformation of conifers generally requires embryogenic cultures as target cells.   The advantage of using such cultures is that they offer a uniform and fast growing population of cells which can develop into embryoids from single cells.

15   It has now been determined that transformation of pine and particularly *Pinus radiata* can be achieved using mature embryos and shoot forming callus as targets.

Using the observation that the shoot meristem of pine is different from its counterpart of dicots and monocots in that

20   it does not have a multiple layer structure and is comprised of a single type of meristematic cell capable of dividing in any direction so as to form a meristematic mass, it has been established that biolistic bombardment is a realistic alternative transformation strategy for such species.   Shoot

25   forming callus of pine has a similar structure to shoot meristem and is amenable to this manner of transformation also.

In the light of the foregoing, there has been established a tissue culture system which regenerates shoots from mature

30   embryos of radiata pine at a consistently high frequency. Shoot forming callus may be induced from shoot tips and bases

of young needles.

The invention will be further described with reference to the following Examples and Figures supporting, illustrating embodiments of the present invention, and wherein:

FIG 1 is expression of the PrMADS1, PrMADS2, PrMADS3, PrFL1 and PrCON1 genes in reproductive and vegetative tissues of *P.radiata*;

FIG 2 is expression of PrMADS1 gene in female cones;

FIG 3 is expression of PrMADS2 gene in female cones;

FIG 4 is expression of PrMADS2 gene in male cones;

FIG 5 is expression of PrMADS3 gene in male and female cones;

FIG 6 is gel mobility shift assay of PrMADS1, PrMADS2 and PrMADS3;

FIG 7 is a promoter finder strategy;

FIG 8 is the nucleotide sequence of PrMADS1 cDNA clone;

FIG 9 is the amino acid sequence of PrMADS1 protein;

FIG 10 is the nucleotide sequence of PrMADS2 cDNA clone;

FIG 11 is the amino acid sequence of PrMADS2 protein;

FIG 12 is the nucleotide sequence of PrMADS2 promoter;

FIG 13 is the nucleotide sequence of PrMADS3 cDNA clone;

FIG 14 is the amino acid sequence of PrMADS3 protein;

FIG 15 is the nucleotide sequence of PrMADS3 promoter;

FIG 16 is the nucleotide sequence of PrFL1 cDNA clone;

FIG 17 is the amino acid sequence of PrFL1 protein;

FIG 18 is the nucleotide sequence of PrFL1 promoter;

FIG 19 is the nucleotide sequence of PrCon1 gene (partial);

FIG 20 is a diagrammatic illustration of a control mechanism to protect non target tissues;

FIG 21 is the plasmid pBR Barnase prom Barstar 1

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containing V1-promoters;

FIG 21A is a diagrammatic illustration of plasmid 7 prom Barstar comprising a promoter and barstar fragment cloned into pGem3zf (Promega);

5   FIG 22 is a diagrammatic illustration of plasmid containing V2-promoters;

FIG 24 is a Northern blot of total RNA extracted from Eucalypt tissue probed with the EGM2 cDNA (3 day exposure);

FIG 25 is a graphical representation of Northern blot of
10   total RNA extracted from Eucalypt tissue probed with the EGM2 and EGM3 cDNA respectively (6 hour exposure);

FIG 26 is a Northern blot of total RNA extracted from Eucalypt tissue probed with the EGM3 cDNA (6 hour exposure);

FIG 28 is the nucleotide sequence of the EGM1 promoter;
15   FIG 28A is a diagrammatic illustration of the EGM1 promoter;

FIG 29 is the nucleotide sequence of the EGM3 promoter;

FIG 29A is a diagrammatic representation of the EGM3 promoter;

20   FIG 29B is the nucleotide sequence of the EGM3 promoter;

FIG 30 is the nucleotide sequence of the EGM2 promoter;

FIG 30A is a diagrammatic representation of the EGM2 promoter;

FIG 31 is a diagrammatic representation of agarose
25   separation of a *Pst1* digest of an EGM3 double bin. Given that the *EcoRI* cloning site is at base 6772 in EGM3 bin, the predicted fragment sizes from the map for this digestion are 4.9, 4.8, 4.4, 3.3, 1.9, 1.1, and 0.6 kb;

FIG 32 is a diagrammatic representation of two gel
30   exposures of a *Pst1* digest of an EGM3 bin showing the other possible orientations of the cloned insert. The sizes of fragments expected from this digest are 7.4, 4.9, 4.4, 1.1, 0.7, and 0.6 kb;

FIG 33 is a plasmid diagram of EGM3 double bin wherein the
35   illustrated fragment is cloned in both orientations;

FIG 34 is a plasmid diagram of EGM3 V1 sense;

FIG 35 is a plasmid diagram of plasmid V1 (Barnase-

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Barstar);

FIG 36 is a plasmid diagram of pBR Barnase;

FIG 37 is a plasmid diagram of plasmid V2 (LacIqNLS-35S promoterOp-Barstar);

5   FIG 38 is a plasmid diagram of p35Sop Barstar E-2;

FIG 39 is a plasmid diagram of p35Sop Barstar, and serves also to represent 35Sop Barstar E, which is the same plasmid with the *EcoRI* site at base508 removed;

FIG 40 is a plasmid diagram of pBRlac;

10   FIG 41 is a plasmid diagram of pBRGUS 1;

FIG 42 is a plasmid diagram of pBRGUS 2;

FIG 43 is a plasmid diagram of Bin 19+EGM3 V1 sense (Orientation 2 as described in the following description of embodiments);

15   FIG 44 is a plasmid diagram of EGM3 V2 sense;

FIG 45 is a plasmid diagram of plasmid pBRLacBH-;

FIG 46 is an illustration of *Arabidopsis* (FB.13L30) transformed with an antisense PrMADS1 gene compared with a control plant; and

20   FIG 47 is a diagrammatic representation of the directed development strategy resulting in the transformed *Arabidopsis* of FIG 46.

EXAMPLE 1

Isolation of cone-specific genes from *P. radiata*

25   Five cone-specific genes displaying strong homology to

Arabidopsis thaliana and *Anthirrhinum majus* floral meristem and organ identity genes were isolated from *P.radiata* cDNA library prepared from immature female and male cones. Three of them, PrMADS1, 2 and 3, belong to the family of MADS-box genes showing homology to *Arabidopsis* AGL-2, AGL-4 and AGL6 genes and *dall* gene from another non-angiosperm, *Picea abies* (Norway spruce), respectively. The PrFL1 gene is the pine ortholog of *Arabidopsis* Leafy (*Lfy*) and *Floricaula* (*Flo*) gene from *Anthirrhinum*. The PrCon1 shows strong homology to *Arabidopsis* CONSTANS (*co*) gene. To elucidate the function of these genes the approach of characterising their expression pattern in male and female cones during different stages of cone development was taken.

A significantly lower level of expression was detected in vegetative tissues: vegetative buds, needles, stem and roots. *In situ* hybridisation showed that expression of these genes is substantially detectable only in reproductive tissue cells.

Expression analysis revealed that all five genes show different patterns of expression in different stages of development of male and female cones (Figs. 1-5). PrMADS1, 2 and 3 genes are cone-specific: expression of both genes was restricted to reproductive organ primordia tissues. No detectable expression of these genes was observed in vegetative tissues: vegetative buds, needles, stems, roots. For PrFL1 and PrCon1 low detectable expression was observed in vegetative buds.

In reproductive organs low level of expression of both genes was detected at early stages of cone development (5 mg cones) which was increased during a cones development (50 mg cones). In male cones expression of both genes was restricted to microsporangia containing primary sporogenous cells. In female cones expression of both genes was restricted to premature ovules.

To characterise MADS proteins as DNA-binding proteins *in*

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vitro, we expressed both proteins in *E.coli* and characterised
their DNA-binding properties. PrMADS1,2,3 proteins are sequence-
specific DNA-binding proteins. Their DNA-binding consensus
sequence is similar to that of the *AGAMOUS* protein. All three

5   proteins bind a DNA sequence matching the consensus sequence of
CArG box TT(A/T)CC(A/T)(A/t)$_2$(T/A)NNGG(-G)(A/T)$_2$ (oligo A) for
PrMADS1 and PrMADS2) (Fig.6). Mutation of these consensus
sequences (oligo B) significantly decreases their binding of
PrMADS 1,2, or 3 proteins. Competition with non-radioactive

10   oligos did not decrease binding of any of the proteins to the CArG
consensus. This indicates that we are dealing with specific DNA-
protein interactions.

EXAMPLE 2

Isolation of promoter sequences of PrMADS2,3 and PrFL1 genes

15   Upstream sequences were isolated using a 'Promoter finder'
strategy (Fig.7). A special adaptor was ligated to the ends of DNA
fragments generated by digestion of genomic DNA from *P. radiata* with
*Eco*RV, *Sca*I, *Dra*I, *Pvu*II and *Ssp*I separately. The enzymes used were
selected because they have six-base recognition sites and generate
blunt ends. Following adaptor ligation, these DNA fragments were
used as a template for PCR using first adaptor primers AP1, AP2 and
gene-specific primers GSP-1,2.

The sequences of adaptor (first sequence), adaptor-primers, and
polymerase blocking primer are shown below. It is noted that adaptor

25   primer AP1 corresonds to bases 1 to 22 of the adaptor, adaptor primer
AP2 corresponds to bases 13 to 31, and the polymerase block
corresponds to bases 41 to 48.

Adaptor: 5'GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3'
Polymerase block: 3'-NH$_2$-CCCGACCA-PO$_4$-5'

Adaptor primer 1. (AP1):   5'-GTAATACGACTCACTATAGGGC-3'

Adaptor primer 2. (AP2):   5'-ACTATAGGGCACGCGTGGT-3'

    The presence of the amine group on the 3' end of the lower
strand blocks polymerase catalysed extension from free adaptor
molecules that have not been ligated, thus preventing the generation
of the primer binding site unless a defined, gene specific primer
extends a DNA strand opposite the upper strand of the adaptor.
    Primary PCR was performed using Avantage Tth Polymerase Mix
(Clontech) and adaptor primer AP1 and GSP-1 for PrMADS2,3 and PrFL1
genes.
    GSP-1 sequences:

PrMADS2: 5' CGGCGCTTCCGAACTCATAGAGTTTTCCTC 3'
PrMADS3: 5'TTAGCGCCACTTCGGCATCGCACAGC 3'
PrFL1:   5" CAAGGGACTTCAAATCCTTTCTCCCATTCATGG 3'.

    PCR two step cycle parameters:

7 cycles: 94°C   25 sec
               72°C   4 min

32 cycles: 94°C   25 sec
               67°C   4 min

67°C for an additional 4 min after the final cycle.
    The 1 ml of primary PCR was used in secondary PCR using the same
cycling parameters and a second set of GSP primers with AP2 adaptor-
primer.

20

GSP-2 primers sequences:

PrMADS2:   5' CGCCTTTTTCAGCAGACCATTCCGGC 3'

PRMADS3:   5' CAGCAGTCCGTTTCCGGCGCTTCG 3'

PrFL1:   5' CGTCCATGGTCCTTGTTAAAGACAGTTGTTGTTGG 3'

5   The 1-2 kb PCR fragments were cloned into the TA-type cloning

vector and sequenced. Sequences of cDNAs, proteins and promoter

regions PrMADS2, PrMADS3 and PrFL1 are shown on Figs 12, 15 and 18.

        EXAMPLE 3

        Introduction of promoter regions into the sterility cassettes V1 and V2

10   One difficulty with the tissue ablation approach is the

effect of promoter leakage or expression in other tissues.   To

overcome this we have developed a control system (gene cascade)

whereby expression of a lethal gene(s) is countered in non-target

tissues (Fig.20).   Two different vectors carrying Barnase (V1) and

15   Barstar plus repressor (lacIq) (V2) parts of these cascade were

designed.

        In order to clone promoter regions into the vectors V1 and V2

they have been amplified using sets of primers with SalI restriction

sites on the ends for V1 (SalI primers) and EcoRI restriction site

20   for V2 (EcoRI primers).   In order to check the orientation of the

promoters in V1 and V2 vectors an EcoRI restriction site was designed

in the forward SalI primer downstream from SalI site and a SalI site

was designed in the EcoRI primer downstream from EcoRI:

SalI primers:

<u>PrMADS2 promoter:</u>

Forward primer:

5' CCG<u>GTCGAC</u><u>GAATTC</u>CGACAGTGGAGTCCACAAAGAAAGATGCG3'

5   *SalI*   *Eco*RI

Reverse primer: 5' CCG<u>GTCGAC</u>TTCTTTCCTTCTTTTCTTTCTGC 3'

                          SalI

<u>PrMADS3 promoter:</u>

Forward primer: 5' ACGC<u>GTCGAC</u><u>GAATTC</u>AAGATTTCAAATCAGTCC 3'

10   *SalI*   *Eco*RI

Reverse primer: 5' ACGC<u>GTCGAC</u>CAAGATCCCTCTGCTTCTTCACC 3'

                       SalI

<u>PrFL1 promoter:</u>

Forward primer: 5' ACGC<u>GTCGAC</u><u>GAATTC</u>GAACTTCTGGAATAAGCTGC 3'

15   *SalI*   *Eco*RI

Reverse primer: 5' ACGC<u>GTCGAC</u>TTCATCTTACGTCACGCGAGG 3'

                    SalI

*Eco*RI primers:

<u>PrMADS2 promoter:</u>

20   Forward primer:

5' CCG<u>GAATTC</u><u>GTCGAC</u>CGACAGTGGAGTCCACAAAGAAAGATGCG3'

         *Eco*RI *SalI*

22

Reverse primer: 5' CCGGAATTCTTCTTTCCTTCTTTTCTTTCTGC 3'

                    EcoRI

PrMADS3 promoter:

Forward primer: 5' CGGAATTCGTCGACAAGATTTCAAATCAGTCC 3'

5   *EcoRI* *SalI*

Reverse primer: 5' GCGAATTCCAAGATCCCTCTGCTTCTTCACC 3'

                    EcoRI

PrFL1 promoter:

Forward primer: 5' CGGAATTCGTCGACGAACTTCTGGAATAAGCTGC 3'

10   *EcoRI*   *SalI*

Reverse primer: 5' GCGAATTCTTCATCTTACGTCACGCGAGG 3'

                    EcoRI

        PCR fragments of *SalI* primers were digested with *SalI*
restriction enzyme and introduced into the V1 vector digested with

15   *SalI*. The orientation of promoters was checked using digestion with
EcoRI enzyme.

        PCR fragments of *EcoRI* primers were digested with *EcoRI*
restriction enzyme and introduced into the V1 vector digested with
EcoRI. The orientation of promoters was checked using digestion with

20   *SalI* enzyme. Maps of the resulting plasmids are shown in Figs. 21
and 22.

        These vectors were linearised with HindIII enzyme and introduced
into the Bin19 binary vector digested with HindIII. Colonies
carrying modified Bin 19 vectors were selected on plates with

25   kanamycin and ampicillin antibiotics. As a next step Bin19-V1-
promoter and Bin19 V2-promoter vectors were transformed into several

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Agrobacterium strains. *Arabidopsis thaliana.* *Eucalyptus grandis* and *Pinus radiata* embryos and explants were co-transformed with Agrobacterium strains.

EXAMPLE 4

5   **Plasmid construction for lethal gene constructs**

The HindIII fragment from the plasmid pRT 99 gus (Töpfer *et al.* Nucleic Acids Research (1988) 16 (17): 8725) was cloned into the HindIII site of pBR 322. This insertion resulted in two plasmids corresponding to the insert being cloned in both

10   orientations. The inserted region contains the Cauliflower Mosaic Virus (CaMV) 35S RNA promoter, beta glucoronidase (GUS) gene, and CaMV 35S terminator region. These plasmids were called pBrGUS1 and pBrGUS2 and provided the basis for the lethal gene constructs. The orientation of the insertion event was checked with a BamHI

15   digest. PBRGUS 2 results in BamHI fragments of 2.5kb and 4.4kb, pBRGUS 1 in fragments of 6.1kb and 0.8kb.

The DNA encoding the lacIq nuclear localisation signal peptide was amplified from the plasmid pGEM lacIqNLS by PCR using the 5' primer Lac I (this has an EcoR1 site) and the 3' primer D23 which has a KpnI site. The amplified DNA fragment was restricted with EcoRI and KpnI and cloned into pBRGUS 2 cut with the same enzymes. The resulting plasmid was called pBRLac. The pBRLac plasmid was then cut with SphI and run on an agarose gel to allow the purification of the plasmid fragment containing the LacIq

25   gene, Ampicillin resistance gene, and origin of replication away from the small SphI fragment, which contained restriction sites that we wished to remove. The resulting plasmid was called pBRLacBH-.

The second stage in the plasmid construction was the

30   preparation for cloning of the Barstar gene under the regulation

24

of the modified CaMV plus lac operator promoter. This was amplified from the plasmid p35S-op-Barstar EcoRI- which contained the promoter/gene sequence which had been modified to remove the EcoRI site between the promoter and coding sequence. This

5 modification was accomplished by cutting the 35S-op-Barstar plasmid with EcoRI, carrying out a blunting reaction with T4 DNA polymerase, and then religating the blunted plasmid. The PCR was carried out using the 5' primer pQE-F and the 3' primer Bar-3'. The PCR fragment was cut with XhoI and KpnI. This was cloned into

10 the 35S-op-Barstar EcoRI- plasmid also cut with XhoI and KpnI, and from which the unwanted gene had been purified away by gel electrophoresis. The removal of restriction sites at the three prime end of the Barstar coding region. This plasmid was called p35S-op-Barstar EcoRI- 2.

15 The Barstar gene was then cut out of the p35S-op-Barstar EcoRI- 2 plasmid with XhoI and SalI and cloned into pBrLac BH- cut with SalI. The Barstar gene could go into the SalI site in either orientation but only one orientation was found. The orientation of the insert was ascertained by a KpnI digest. Only a 1kb band

20 was seen which was indicative of the orientation of the insert seen in plasmid pBRLac Op Barstar 1. The resulting plasmid was called V2.

The plasmid was then ready for the cloning of, in this case, a flower specific promoter, into the unique EcoRI cloning site 5

25 prime of the lacIq gene. The EGM3 promoter from the Eucalyptus MADS gene EGM3 was used for this purpose. This was cut from plasmid pEGM3 with EcoRI. This promoter was cloned in both orientations, and the resulting plasmids were called V2 EGM3 sense and V2 EGM3 antisense. The plasmid V2 EGM3 sense was used as a

30 source the EGM3 regulated lacIq and CaMVop Barstar genes for the plant transformation vector. The orientation of the EGM3 promoter

25

was determined by an XbaI PstI digest. The presence of a 2.3 and 0.9 kb band was indicative of the correct orientation.

The second construct V1, containing the promoterless Barnase gene, was constructed using pBrGUS 2 as the starting point. A
5   Barnase gene was amplified from genomic Bacillus amyloliquefaciens using the primers Barnase 5 prime Sal, and Barnase 3 prime Kpn. The resulting PCR product was restricted with Kpn I and Sal I as was pBRGUS 1 and a ligation reaction performed. The plasmids from the resulting colonies were used as templates for sequencing using
'0   the amplification primers. Plasmid SB4 was found to contain a Barnase fragment of the same sequence as the B. amyloliquefaciens Barnase gene and this was called pBRBarnase and this was used for further constructions.

Previous work using Barnase as a lethal gene in plants had
15   shown that the anti-toxin gene for Barstar was required to be present and expressed from the same plasmid as the Barnase before a promoter region could be cloned 5 prime of the Barnase gene (Paul et al. 1992 Plant Molecular Biology 19: 611-622). This was achieved by using a plasmid that contained the Barstar gene and
20   promoter from B. amyloliquefaciens in cis with the Barnase sequence. To this end the promoter plus Barstar DNA region was amplified from the B. amyloliquefaciens genomic DNA using primers 5 prime Barstar promoter and Bar 3 prime. The PCR fragment was restricted with KpnI as was pGEM 3f and a ligation reaction
25   performed. The resulting white colonies were screened for inserts. The DNA from colony 7 was used for sequencing and found to contain the Barstar promoter plus Barstar sequence as in the genomic B. amyloliquefaciens DNA. This plasmid was call 7 prom Barstar, as shown in Fig 21A.
30   The 7 prom Barstar plasmid was restricted with Kpn I as was pBRBarnase and the Promoter Barstar fragment was cloned into the

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Kpn I site of the pBRBarnase DNA. This resulted in a plasmid known as V1. The EGM3 promoter was cut out of pEGM3 using EcoRI, blunted using DNA polymerase I (Klenow fragment) and cloned into the unique SalI cloning site of the V1 DNA which had also been

5 restricted and blunted. The resulting plasmid with the promoter inserted in the correct orientation was called EGM3 VI sense.

The EGM3 V1 sense DNA was linearised with HindIII and cloned into the plant transformation vector Bin19 also linearised with HindIII. This resulted in two plasmids, EGM3 V1 Bin 1 and 2,

10 corresponding to the two orientations of insertion. In orientation 2, the two EcoRI sites present in EGM3 V1 Bin are approximately 80 bp apart. The EGM3 V1 Bin 2 was cut with EcoRI and blunted using DNA polymerase I (Klenow fragment). The EGM3 promoter lacIq gene, and the CaMV 35S op Barstar genes were cut

15 out of EGM3 V2 sense using AatII and HindIII. This fragment was also blunted and ligated into the EcoRI cut and blunted EGM3 V1 Bin 2 to create the plasmids EGM3 Double Bin I and II, again corresponding to the two possible orientations. These plasmids were used for transformation of plasmids.

20 The procedure was then repeated with further *Eucalyptus* MADS promoters. These were EGM2 long and short, and a shorter version of EGM3.

Primers.

Barnase 5 prime Sal

25 5' CC<u>GTCGAC</u>ATGGCACAGGTTATCAA 3'
        SalI

Barnase 3 prime Kpn

5' CG<u>GGTACC</u>TTATCTGATTTTTGTAAAGG 3'
        KpnI

27

Five prime Barstar promoter

5' CG<u>GGTACC</u>GTCCAATCTGCAGCCGTCCGA 3'

      KpnI

Bar 3'

5   5' GC<u>GGTACC</u>TTAAGAAAGTATGATGGTG 3'

       KpnI

D23

5' CG<u>GGTACC</u>TATCTCCTATTTCCCCCACG 3'

       KpnI

10   pQE-F

5' GCGTATCACGAGGCCCTTTC 3'

LacI

5' GC<u>GAATTC</u>AACATGGAACCAGTAACGTTATA 3'

          EcoRI

15   <u>EXAMPLE 5</u>

    Expression of the three *Eucalyptus* MADS-box genes in a range
of tissues within two *Eucalyptus* species

    Northern blots were performed on a range of tissues in order
to determine the tissue specificity of the expression of the three

20 EGM MADS-box genes. Tissue used for isolation of RNA from roots,
seedlings, stems, shoots, leaves and mature flowers was obtained
from <u>Eucalyptus grandis</u> plants. Tissue for isolation of RNA from
floral tissues including receptacles, petals, stamens, carpels,
and styles was collected from *E. globulus* flowers. This species

25 was used because it produces very large flowers, making collection
of sufficient amounts of RNA much easier. Ten micrograms of total

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RNA was used in the blots which included the different floral
tissues and 20 micrograms was used in blots of *E. grandis* RNA
which included the vegetative tissue. Blots were probed with the
three EGM genes which had been digested with the appropriate

5   restriction enzymes to remove the MADS-box.

The northern blots indicated that all three EGM genes are
expressed at a high level in eucalypt flowers. When northern
blots are exposed to film for long periods, weak expression of the
EGM2 and EGM1 genes was detected in floral tissue. The EGM3 gene

10   was specifically expressed in vegetative tissue. Within the
flower, the EGM2 gene was observed to be expressed in stamens and
petals. The EGM3 gene is expressed in receptacles, petals,
stamens, carpels and styles.

EXAMPLE 6

15   DIRECTED DEVELOPMENT STRATEGY

This strategy involves the expression of an antisense version
of a gene critical to the development of reproductive structures
so that vegetative development occurs instead.

The MADS-box region of PrMADS1 gene was fused in the frame to
the amino end of GUS reporter gene's coding region in sense and
antisense orientations (Fig. 47). Constitutive expression of
these genes will lead to high level of accumulation of the
corresponding products in cytoplasm and nucleus. Accumulation of
the fusion proteins containing sense orientation of the MADS-box

25   region could lead to the inhibition of downstream transcription
processes through competitively binding to specific trans-acting
elements in the promoter regions.

Antisense orientation could block expression of MADS-box
genes through RNA-RNA interactions between fusion antisense MADS-

30   GUS mRNA and target MADS-box mRNAs in floral meristem.

29

Constructs were introduced into the binary vector Bin19 and transformed into *AGL1* Agrobacterium strain. *Arabidopsis thaliana* plants were transformed using a root transformation procedure and transformants were selected on medium containing kanamycin antibiotic. Only antisense constructs were analysed for floral inhibition.

The 25-30% of transformants with antisense MADS-box genes (lines FB13L) did not induce any inflorescence even 45 days after transferring to the soil. These plants look very bushy with much larger leaves than controls (Fig. 46). After 45 days control plants had already passed through all vegetative stages and finally gave seeds. Usually, in *Arabidopsis* plants floral buds have already appeared when 8-10 rosette leaves are appearing.

In FB13L plants there was no detectable floral induction even after 25-35 leaves. Leaves of FB13L line plants were stained with X-gluc (histological GUS assay). Most of the plants have shown the blue color of the leaves which indicates a high level of accumulation of GUS proteins.

EXAMPLE 7

Transformation of *Eucalyptus*

A number of abbreviations are used in the following text. These are in common use in the field of plant tissue culture.

BA: benzyladenine also known as 6-benzylaminopurine.

IBA: indole-3-butyric acid.

NAA: 1-naphthaleneacetic acid

TDZ: thidiazuron also known as 1-phenyl-3-[1,2,3-thiadiazol-5-yl] urea.

The following is a detailed description of the preferred steps for producing transgenic *Eucalyptus* from shoot and seedling

30

explant material.

Shoot explants

1. Subculture shoots monthly on solid KG medium containing
0.2mM BA. Keep in low light (16 hour photoperiod, 100-350 Lux or
5   1-8 mmolm^{-2}s^{-1} PAR) and 22.5°C.

2. Use whole shoots 3-4 weeks after subculture.

3. Remove lower leaves leaving the top 4-6 leaves.

4. Wound leaves 5 to 6 times with a needle (e.g. 25G) and
place whole shoots in an *Agrobacterium* suspension (approximately
10   1x10^8 cfu ml^{-1}) containing 10-100mM acetosyringone for 10 minutes
to 2 hours. An *Agrobacterium* suspension with an optical density of
1.0 at 600nm diluted 1/20 is approximately 1X10^8 cfu mL^{-1}. Best
results when wound near base of leaf. Alternatively, instead of
leaving the wounded shoots in an *Agrobacterium* suspension for 1
15   hour, the shoots can be vacuum-infiltrated with the *Agrobacterium*
for 10-30 mins at 40-100 Kpa. The shoots do not need to be wounded
for the vacuum infiltration procedure. However, callus formation
is greater when wounding is effected with a needle rather than by
vacuum-infiltration.

20   5. Blot shoots between sterile filter papers and insert
shoots vertically into KG medium containing 0.2mM BA. Co-culture
for 2 days in the dark.

6. Transfer shoots (still upright) to KG medium containing
0.2mM BA and 200 mgL^{-1} cefotaxime. Keep in low light for 5 days
25   (16 hour photoperiod, 100-350 Lux or 1-8 mmolm^{-2}s^{-1} PAR).

7. Excise 4-6 upper leaves and place them, adaxial face up,
on solid callus induction medium G22 containing 2mM BA, 2.5mM NAA,
200 mgL^{-1} cefotaxime and 5-10 mgL^{-1} geneticin. Incubate for 2 weeks
in the dark.

30   8. Transfer explants to G22 medium containing 2mM BA, 2.5mM
NAA, 1.0mM TDZ, 200 mgL^{-1} cefotaxime and 15 mg.L^{-1} geneticin.

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Subculture every 2 weeks in the dark. Brown phenolic compounds are produced when incubated in the light.

9. After 6 weeks transfer explants to shoot induction medium GBA (i.e. G22 containing 5mM BA and 0.5mM NAA) containing 200 mgL^{-1} cefotaxime and 15 mgL^{-1} geneticin. Leave in dark for 5-6 days then move into light (16 hour photoperiod, 100-350 Lux or 1-8 mmolm^{-2}s^{-1} PAR). Subculture every 2 weeks.

10. After 8-10 weeks on GBA with 200 mgL^{-1} cefotaxime and 15 mgL^{-1} geneticin transfer pieces of callus with buds and callus formed on the original explants to GBA with 200 mgL^{-1} cefotaxime and 30 mgL^{-1} geneticin. Subculture every 2 weeks.

11. After 4-6 weeks on this medium place regenerated shoots in liquid KG medium containing 0.01 mM BA, 50 mgL^{-1} cefotaxime and 5 mgL^{-1} geneticin for 2 weeks then transfer to medium with higher geneticin (10 mgL^{-1}) for 2-4 weeks. The liquid cultures are shaken at 100-120 rpm with 8 ml of liquid in a 70 ml container.

12. Transfer surviving shoots and callus to solid medium (KG containing 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin but with no hormones) and higher light intensity (16 hour photoperiod, 450 Lux or 10 mmolm^{-2}s^{-1} PAR). Subculture every 2 weeks.

13. Assay putative transformed shoots for marker gene(s) activity.

14. Regenerate plants from the confirmed positive shoot material. Induce rooting by transferring shoots onto KG containing 10 mgL^{-1} geneticin but with no hormones. However, if there is no rooting after three weeks then move to the same medium containing IBA 0.2 mgL^{-1} (9.8 mM).

Seedling explants

1. Disinfect seeds and germinate on KG containing 0.2 mM BA.

2. Using 10-12 day old seedlings, remove roots and place them in an overnight-grown *Agrobacterium* suspension (approximately

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1x10^8 cfu ml^{-1}) containing 50mM acetosyringone. An *Agrobacterium* suspension with an optical density of 1.0 at 600nm diluted 1/20 is approximately 1X10^8 cfu mL^{-1}. Wound the cotyledons and hypocotyl by gently stabbing with a 30 gauge syringe needle under a dissecting microscope. Incubate for 1 hour then remove the seedlings from the suspension and blot them between sterile filter papers to remove excess liquid. Instead of leaving the wounded cotyledons in an *Agrobacterium* suspension for 1 hour, the cotyledons can be vacuum-infiltrated with the *Agrobacterium* for 20 mins at 95 KPa (28mm Hg). The cotyledons do not need to be wounded for the vacuum infiltration procedure. However, the hypocotyls require wounding even when using vacuum infiltration.

3. Co-cultivate on KG medium (containing 0.2 mM BA) for 2 days in the dark making sure that the seedlings are standing upright in the medium.

4. Transfer the seedlings (still upright) to KG medium (containing 0.2mM BA) and containing 200 mgL^{-1} cefotaxime. Continue incubation in low light (16 hour photoperiod, 100-350 Lux or 1-8 mmolm^{-2}s^{-1} PAR) for 5 days.

5. Excise the hypocotyls and cotyledons. Transfer the hypocotyls to callus induction medium G22 containing 0.5mM BA, 1.0mM NAA, 1.0mM TDZ, 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin. Transfer the cotyledons to G22 medium containing 1.0mM BA, 1.0mM NAA, 0.3mM TDZ, 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin. Continue incubation in the dark for 2 weeks.

6. Transfer explants to the same medium containing 15 mg.L^{-1} geneticin and 200 mg.L^{-1} cefotaxime. Continue dark incubation. Subculture every two weeks until about 6 weeks have elapsed.

7. After 6 weeks, transfer to shoot induction medium GBA (i.e. G22 containing 5mM BA and 0.5mM NAA) containing 15 mgL^{-1} geneticin. Leave the cultures in the dark for 5-7 days then

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transfer them to the light (16 hour photoperiod, 100-350 Lux or 1-8 mmolm^{-2}s^{-1} PAR).

8. After about 10 weeks on the GBA with 15 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime, a number of explants will have produced
5   shoots. These shoots are excised and transferred to KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime. Meanwhile, the callus that has also formed on the original explants is subcultured back to GBA with 15 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime and left for a further month,
10   by which time more shoots may develop which can then be transferred to KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1} cefotaxime.

9. All shoots and callus are now transferred to fresh KG medium (containing 0.2mM BA) with 30 mgL^{-1} geneticin and 200 mgL^{-1}
15   cefotaxime for another month or so.

10. After 4-6 weeks on this medium place regenerated shoots in liquid KG medium containing 0.01 mM BA, 50 mgL^{-1} cefotaxime and 5 mgL^{-1} geneticin for 2 weeks then transfer to medium with higher geneticin (10 mgL^{-1}) for 2-4 weeks.
20   11. Transfer surviving shoots and callus to solid medium (KG containing 200 mgL^{-1} cefotaxime and 10 mgL^{-1} geneticin but with no hormones) and higher light intensity (16 hour photoperiod, 450 Lux or 10 mmolm^{-2}s^{-1} PAR). Subculture every 2 weeks.
25   12. Assay putative transformed shoots for marker gene(s) activity.

13. Regenerate plants from the confirmed positive shoot material. Induce rooting by transferring shoots onto KG containing 10 mgL^{-1} geneticin but with no hormones. However,
30   if there is no rooting after three weeks then move to the same medium containing IBA 0.2 mgL^{-1} (9.8 mM).

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Seedlings of 12 days old or younger are best for transformation but seedlings up to 20 days old can be used for regeneration.

Shoot regeneration frequencies for non-transformed cotyledon and hypocotyl explants without selection are approximately 80%.

Experience has shown that removal of cefotaxime from the medium, even after 3 months, will result in rapid overgrowth by Agrobacterium. The concentration of cefotaxime varies between liquid and solid media.

Shoot material grew faster in liquid medium and formed more shoots when compared to shoots grown on solid medium. This liquid selection step has the advantages of reducing false positives by increasing selection pressure, reducing residual Agrobacterium and increasing the amount of shoot tissue compared to solid grown cultures.

The regenerated rooted transgenic plants are then moved to a soil based medium and grown into trees of a size and form suitable for planting. Clones can be micropropagated by tissue culture propagation techniques and grown into trees of a size and form suitable for planting.

The media used in this study were G22, GBA and KG as described by Laine and David (1994). However, various basal media, including MS, B5 and P24, have been tested and found to support shoot regeneration. The plant growth regulator regimes were generally different from those of Laine and David (1994), which were in the combination of 1-3 mM BA, 0.05-2 mM TDZ and 0.5-2.5 mM NAA for callus induction from leaves; 1-3 mM BA, 0.05-1 mM TDZ and 0.5-2.5 mM NAA for cotyledons; and 1-3 mM BA, 0.05-2 mM TDZ and 0.5-2.5 mM NAA for hypocotyls. The differentiation medium was generally a

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GBA medium, which was a G22 but supplemented with 2.5 - 5 mM
BA and 0.5 mM NAA (Laine and David, 1994). A KG medium
containing 0.2 mM BA is generally used as subculture medium
for clone materials. All media were solidified with 0.25%
Gelrite or Phytagel. pH was adjusted to 5.7 - 5.8 using
potassium hydroxide before autoclaving for 15 minutes at 121

C.

A preferred method for the preparation of Agrobacterium
inoculum is described below.

1. Streak out the Agrobacterium strain containing the
construct onto plates with selection, eg YEP + rifampicin
(50 mg/L) + kanamycin (100 mg/L) for LBA4404, EHA105 and
AGL1 containing the pBin GUSINT construct.

2. Incubate plate at 28oC for 2 days.

3. Pick a single colony into YEP broth with selection
and grow on shaker overnight at 28oC.

4. Use the overnight culture to inoculate (1% inoculum)
fresh medium with selection and grow on shaker overnight at
28oC. This additional step will produce a more consistent
inoculum for plant transformation.

5. Harvest Agrobacterium, wash and resuspend in tissue
culture medium. Dilute to an appropriate concentration ready
for transformation.

6. Streak out the resuspended Agrobacterium on to
lactose yeast medium plates and incubate plate at 28oC for
2 days. Do a Benedict's test (Bernaerts and Deley, 1963) on
the colonies to confirm they are Agrobacterium.

This strategy will give good thick cultures of
Agrobacterium on the day of transformation. A culture grown
overnight which was inoculated from a plate or glycerol
stock will give variable results.

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An AGL1 culture grown as described above and diluted to an optical density (600nm) of 0.98 had a viable count of 2.37×10^9 cfu/ml.

It will of course be realised that while the above has been given by way of illustrative example of this invention, all such and other modifications and variations thereto as would be apparent to persons skilled in the art are deemed to fall within the broad scope and ambit of this invention as is herein set forth.

CLAIMS

1.   A method of enhancing vegetative growth in a plant including the steps of:-

identifying a gene having a substantially tissue-specific promoter expressing during the development of both male and female plant reproductive structures;

constructing an expression cassette comprising a heterologous coding region capable of expressing a product which aborts said development under the expression control of said promoter;

transforming plant cells with said expression cassette, and

selecting and vegetatively propagating the transformants.

2.   A method of enhancing vegetative growth according to Claim 1, wherein said gene is selected from genes expressing specifically in the development of both male and female reproductive structures.

3.   A method of enhancing vegetative growth according to Claim 2, wherein said heterologous coding region expresses a lethal gene product selected from lethal translation products and antisense RNAs.

4.   A method of enhancing vegetative growth according to Claim 1, wherein said promoter directs expression of said heterologous coding region substantially in said both male and female reproductive structures, and wherein the effect of leakage of said promoter in non target tissues is suppressed.

5.   A method of enhancing vegetative growth according to
Claim 4, wherein said heterologous coding region expresses a
lethal gene product selected from lethal translation
products and antisense RNAs.

6.   A method of enhancing vegetative growth according to
Claim 5, wherein said suppression is provided by including
in said expression cassette a control gene cascade
functioning to prevent expression of, or counter the
expressed product of said heterologous coding region.

7.   A method of enhancing vegetative growth according to
Claim 6, wherein said control gene cascade comprises a
control gene selected to express a gene product acting to
prevent expression of or counter the expressed product of
said heterologous coding region, said control gene
expressing under the control of a constitutive promoter
which is itself under the control of a repressor gene the
expression of which is promoted by a second tissue specific
promoter.

8.   A method of enhancing vegetative growth according to
Claim 7, wherein said heterologous region codes for the
enzyme Barnase, said control gene expresses Barstar under
the control of a constitutive promoter including a LacIq op
site, and said repressor gene expresses LacIq under the
expression control of the same or another promoter specific
to the same tissue as said heterologous coding region.

9.   A method of enhancing vegetative growth according to
claim 1, wherein said heterologous coding region expresses

under the control of a tissue specific promoter derived from
the MADS-box genes of *Pinus radiata*, *Arabidopsis thaliana*,
or *Eucalyptus grandis*, or strong homologues thereof of other
species.

10. A method of enhancing vegetative growth according to
Claim 9, wherein said plant is *Pinus radiata*, and wherein
said tissue specific promoter is selected from PrMADS2,
PrMADS3, PrFL1 or PrCon1.

11. A method of enhancing vegetative growth according to
Claim 9, wherein said plant is *Eucalyptus grandis*, and
wherein said tissue specific promoter is derived from the
EGM3 gene.

12. A method in accordance with Claim 1, wherein said
tissue specific promoter is identified from genomic DNA
using a 'promoter finder' strategy (Clontech).

13. A method in accordance with Claim 12, wherein said
identified cDNAs are selected for the presence of a single
gene expressed in both male and female buds.

14. A method in accordance with Claim 13, wherein said
promoter or, preferably, a constitutive is fused with a
heterologous coding region which codes for an expression
product of which is the antisense version of said gene.

15. The plasmid pBR Barnase prom Barstar 1 yielding V1-
promoters according to FIG 21.

16.   The plasmid pBR 35SPromOpBarstar yielding V2 promoters according to FIG 22.

17.   The plasmid EGM3 double bin according to FIG 33.

18.   The plasmid EGM3 V1 sense according to FIG 34.

19.   The plasmid V1 (Barnase-Barstar) according to FIG 35.

20.   The plasmid pBR Barnase according to FIG 36.

21.   The plasmid V2 (LacIqNLS-35S promoterOp-Barstar) according to FIG 37.

22.   The plasmid p35Sop Barstar E-2 according to FIG 38.

23.   The plasmid p35Sop Barstar according to FIG 39.

24.   The plasmid pBRlac.

25.   The plasmid pBRGUS 1.

26.   The plasmid pBRGUS 2.

27.   The plasmid Bin 19+EGM3 V1 sense.

28.   The plasmid EGM3 V2 sense.

29.   The plasmid pBRLacBH-.

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Figure 1

Figure 2

Figure 3

Figure 4

5/50

female 50mg control

female 5mg control

male 5mg control

female 50mg

female 5mg

female 5mg

male 5mg
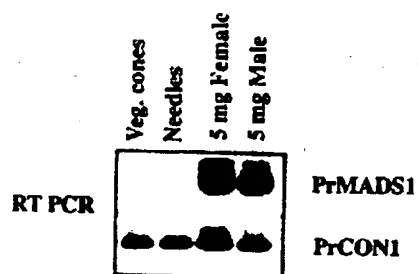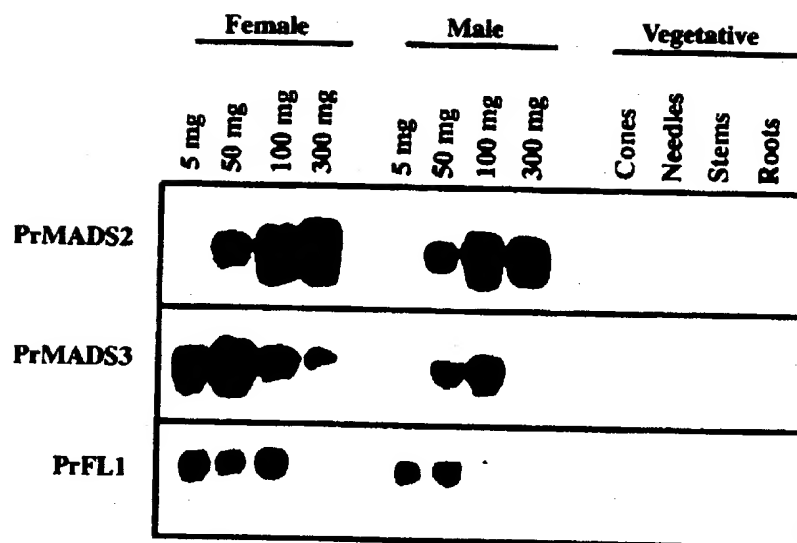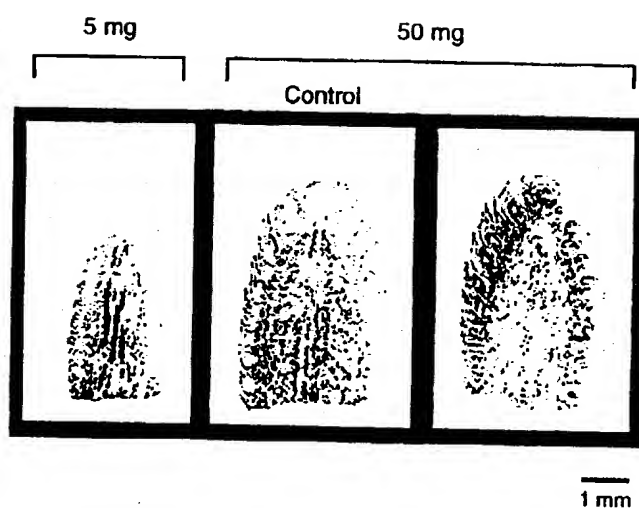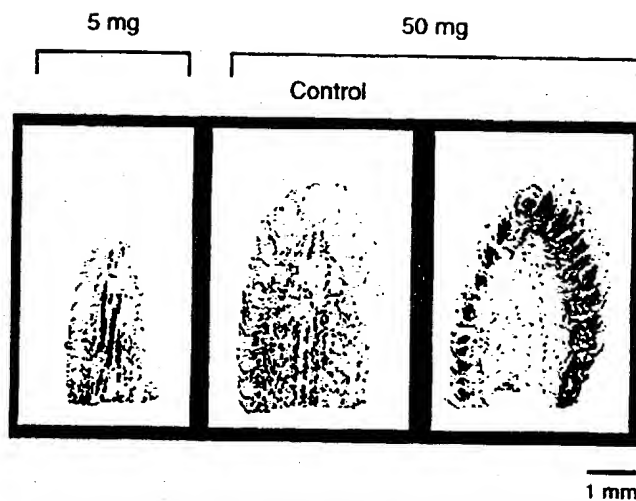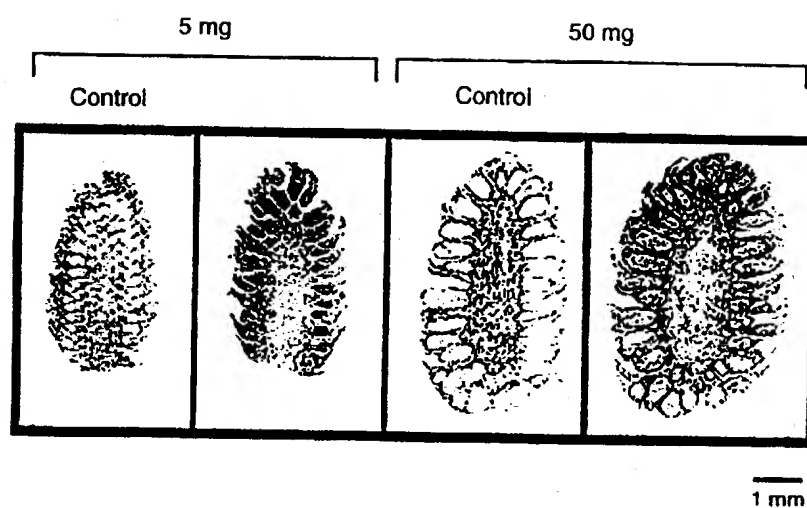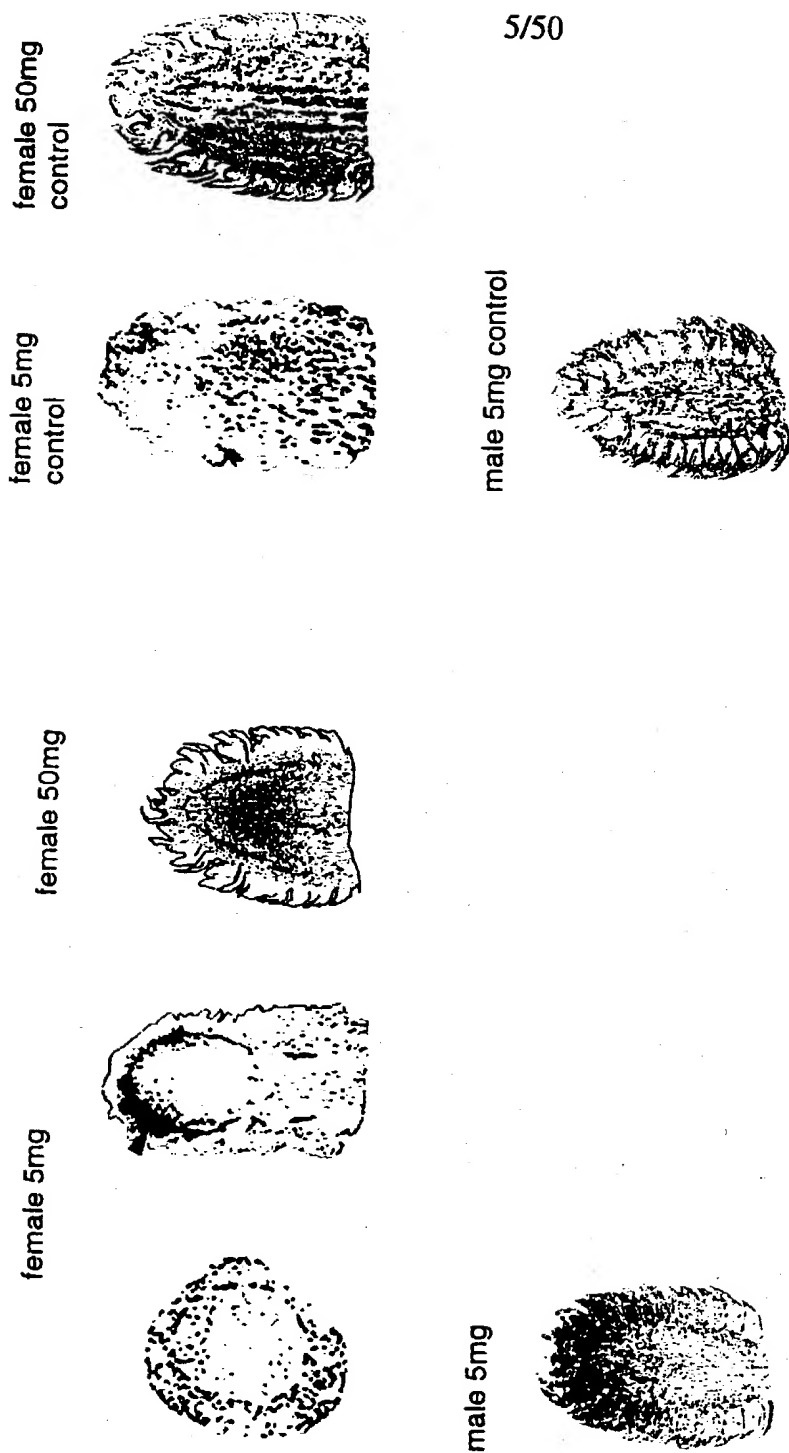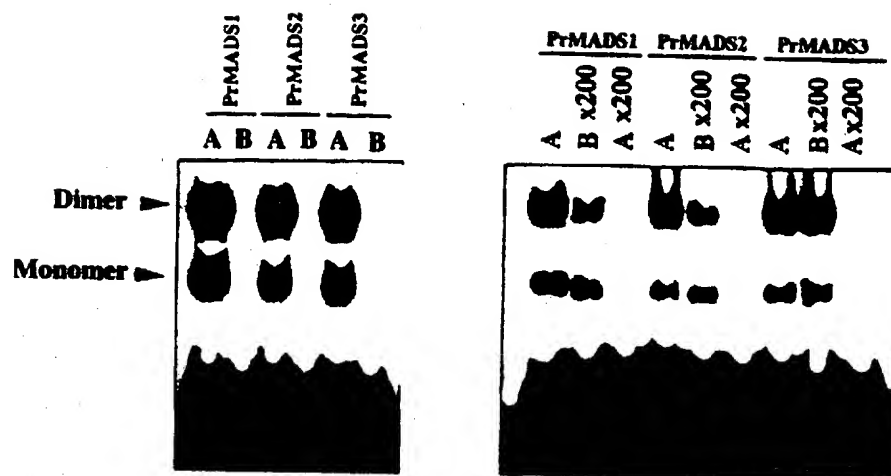
Figure 5

AG consensus CArG-box
NTT(A/T)CC(A/T)NNGG(-G)(A/t)2N

A: TTACCAAAAAAGGAAA

B: TTAGGAAAAAACCAAA

Figure 6

7/50
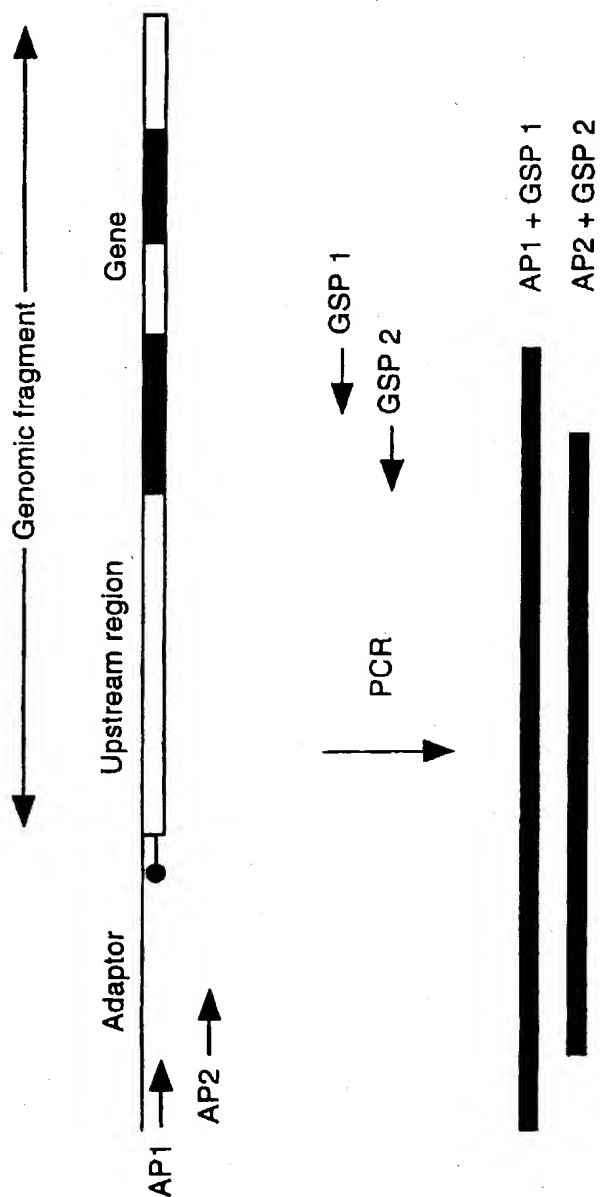
Figure 7

TGAGAAAGAGAGACAGAGATATGGGAAGAGGGAAAGTAGAGCTGAAGAGGATAGAGAA

CAAAATCAACAGGCAAGTAACATTTGCGAAGAGAAGAAATGGGCTTCTCAAGAAAGCTT

ATGAGCTCTCTGTTCTCTGTGATGCTGAGGTTGCGCTCATCATTTTCTCCAACCGTGGCAA

GCTCTATGAATTCTGCAGCAGTTCTAGCATGATGAAAACAATTGAGAAGTACCAGAAGTG

CAGCTATGGTTCACTTGAGAcCAACTGCTCCATCAATGAGATGCAGAACAGCTACCAGGA

TTATTTGGAGCTAAAAGCAAGAGTGGAGGTCCTCCAACGATCTCAGAGAAACCTCCTTGG

GGAAGAGTTGGGTCCCCTAAACTCGAAGGAGCTGGAGCAACTTGAGCACCAGTTGGAG

AATTCTCTGAAGCAAATTCGGTCTGCAAAGACCCAATTCATGTTTGATCAACTGGCTCATC

TTCAGCACAAGGAACAAATGCTGGTTGAAGCTAACAGAGAATTAAGGAAGAAGTTGGAA

GAGAGCAATACAAGAATCCCTCTCCGCCTTGGATGGGAAGCTGAGGATCACAATAACAT

TTCATACAGGCGCCTTCCCACGCAGTCGCAAGGATTGATCTTCCAGCCCTTAGGCGGCTA

CCCGAACATGCAGATCGGGTACAATCCTGCAGGCTCGAATGAATTGAATGTTTCGCCTGC

CGACCAACATCCCAacGGATTCATTCCCGGATGGATGCTCTGAATCGTTCCGCAAGTGAA

CTGCTTGCTGGAAGTTCCATATCAAGTACATTTTCCGGTTTTTGCTATGATATATGACTCTT

CTTCTTCTGGATGACCTATACGAAGATCCATCATTCGTGGATATTGTCCATGGACGTACCC

TAAAAGGAAGGACGGTATGAATCCAATCTAGCTTACTATTTTGTATAAGAATAAAGATCT

GTGCTGCTGATATTTGGAATTCATCTATGTTATTTAATGTATGGAATCC

Figure 8

MGRGKVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCSSSS

MMKTIEKYQKCSYGSLETNCSINEMQNSYQDYLELKARVEVLQRSQRNLLGEELGPLNSKEL

EQLEHQLENSLKQIRSAKTQFMFDQLAHLQHKEQMLVEANRELRKKLEESNTRIPLRLGWEA

EDHNNISYRRLPTQSQGLIFQPLGGYPNMQIGYNPAGSNELNVSPADQHPNGFIPGWML

Figure 9

GAATTCGCGGCCGCTAGAAGAATAAGGAAGAGGCCGAGAGAGGGAGCATTATCGTCGA

GCAGGAGGGGGAAAGAAAGAAAGAAAGAAGGAAAGAATGGGGCGAGGGCGCGTCGAG

CTGAAGCGGATCGAGAATAAGATTAACCGCCAGGTCACGTTTTCGAAACGCCGGAATGG

TCTGCTGAAAAAGGCGTATGAACTTtCAGTGTTATGTGATGCAGAGGTAGCACTGATaATA

TTCTCAAGCAGAGGAAAACTCTATGAGTTCGGAAGCGCCGGGATGCTCAAGACTCTGGA

GCGATATCAAAAATGTTCATACGTATTGCAAGACGCGACTGTATCGgACCGGgAGGCGCA

GAATTGGCATCAAGAGGTtgGcAAATTAAAAGCCAGAGTTGAACTTTTACAACGATCACAA

AGGCACTTATTAGGTGAAGACCTGGGCCCCTTGAGTaTTAAGGAGCTGCAACAACTGGAA

CGTCAACTTGAGGTTGCACTGACACATGTTAGGTCAAGAAAGACTCAAGTCATGTTGGAA

ATGATGGATGAACTACGCAGAAAGGAGCGAATTTTACAAGAAGTAAACAAATCTCTGCG

CAAGAAGTTGCAGGAGGCCGAGGGACAGGCATTCAATGCCATGCAACCTCCCCCTCATG

CATGGGATTCGCATGCAGTAGCAAATAATGCATATGCTATGCAACATCCATCAAATGCTG

TGGATTGTGAGCCCACATTGCAGATTGGATACCAaTATGCCCCTCCTGAGTCAAGCATGC

CTAGGCATGAACAAGCACAAAACAATTACATGCAAGGCTGGATGGTATAATGTACCACCA

ATTTCTAATGACAAGCAGAAGTATTAATATTTATAAATATTAAAATGTCTGTATATATAGCT

ATGAATTTACAGTTGTATTGTACTTCCATTTTAATTCATGTTTTTCAGTAGTTTGATTGTAG

TAACTTAACATTTTATTTTTCCCAAACCCTAACAACTTTGACTTTTAAAAAGTCACAATTTTA

TGCAACAATATTTGTGTTTATAtATATAATTGCTTTTCCTGTTTAAAAAA

Figure 10

MGRGRVELKRIENKINRQVTFSKRRNGLLKKAYELSVLCDAEVALIIFSSRGKLYEFGSAGM

LKTLERYQKCSYVLQDATVSDREAQNWHQEVGKLKARVELLQRSQRHLLGEDLGPLSIKEL

QQLERQLEVALTHVRSRKTQVMLEMMDELRRKERILQEVNKSLRKKLQEAEGQAFNAMQPP

PHAWDSHAVANNAYAMQHPSNAVDCEPTLQIGYQYAPPESSMPRHEQAQNNYMQGWMV

Figure 11

CTCCCGGCCGCCATGGCCGCGGGATACTATAGGGCACGCGTGGTCGACGGCCCGGGCT

GGTAAAAGACGTCCCTCCGATGAATTCGACAGTGGAGTCCACAAAGAAAGATGCGTACG

CTGAATGCCACGTGTCTATAGTTTGCCCACACCTTTCAAACATACTCTGTACGCAGATCG

GCGGACCTGAAAATTTTCTTCGTATAAATCGTCTGTGTTAAGGATATTTGACCATCGGTCC

GAAAGATGCGACTGTCCGACTGCATCTCGTCTCTGGTAATGTTTTTTTTCAAATTTATAAA

GATTGTTTTGCATTATTATTATTTATCATGTTGCGTAAgATCTGGTGAGCAGTATTATTTGT

CACTTTGTGTGGAATCCCTTATGAAAATATATATTTATtCCAGAAAAgTATTTTAGCTTGTCA

AATTGTGTGGAATCTGGTATAAGTACCTGTATTTATCAGACGTATTATTTGTTAAATTGTG

TGGAATCTGGTATAAATTTATATACTTCGTGAGGAAATGTATTTATTAGCAGTATTACTGG

TCAAACAGAGTGGAATCCTGTATAAATTTATCATATTTAGGAACAAATGTATTTATCAGCG

TTATTATCTGTCAAAATGTGTGGAGACGCGATGACTTTAATTTTTTGATAATAGGCGGAGC

GTATAGGTACGCTGTAGTATTGTAGACTGGCATGTAGAGTACAAAGTTTAGATATTAACC

TAGATTATTTTTGCTATTCGCAGGCTATCGCGTACTCATGGTGAAATGCGTACAGTTTCAA

AGTAAATGGTTGATGTTGAGTGCGGTGGCGGGGGGCAGACACACAAATAGGAATCGGAgA

gCGAGAGTACTGCAGCAATTGTTGTTTAcTTTTTGAGCAAGACGAGGATCAAAGAAGAAT

AAGGAAGAGGCCGAGAGAGGGAGcATTATcGTCGAGCAGGAGGGGGAAAGAAAGAAAG

AAAGAAGGAAAGAATGGGGCGAGGGCGCGTCGAGCTGAAGCGGATCGAGAATAAGATT

AACCGTCAGGTCAcGTTTTCGAAACGCCGGAATGGTcTGCTGAAAAAGGCG

Figure 12

ATGGGGCGGGGTCGGGTTCAGCTGAGGCGAATAGAAAACAAAATAAATCGACAAGTCA

CGTTTTCGAAGCGCCGGAACGGACTGCTGAAgAAgGCGTACGAGCTATCAGTGCTGTGC

GATGCCGAAGTGGCGCTAATAATTTTCTCTACCAGAGGAAAGCTTTACGAGTTTGCCAGT

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ACAGGCGTTTCGGACCGGGAAGCACAGAATTGGCACCAAGAAGTTACAAAGTTGAAGGg

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TAACTGGCATAgAGCAAACTTCTAATACTAATACTGGTACTAACGGTCCTTGGGATTCTTC

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CATGTGGACTGTGAACCCACGCTACAGATTGGTTATCAGCCTGTGGCTCCTGAAAGCATC

GTCCCTCCTCATCAGCCGCCGCACAACCAAACGCCGAAcCAATACATGCAAGGATGGTG

GGTTTGATATTTAACATTTATCATTATCAGTTACTTCAATCACAACAAAAGCCCAAAGCGT

GGTAAATTACGAAATTAGAATTATATTATCATTAAAAAAAAAACCCTATTTTCATTGTATAgC

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TATATTAGACCTTCTTGTCGACAAAGTTTAATTGCATAAATCTTGTATGCTAATCTGGCCG

CTAAAAGAgCGATGGAAAAATAGTTGTCCCATTCACAACACATGATATGTTTAAATCCAAC

GTGTATGTGTCTGCAAAATATTATTATACACTACGGTTTATCAAAAAAAAAAAAAAAAAAA

Figure 13

MGRGRVQLRRIENKINRQVTFSKRRNGLLKKAYELSVLCDEAVALIIFSTRGKLYEFASSSM

NKTLERYEKCSYAMQDTTGVSDREAQNWHQEVTKLKGKVELLQRSQRHLLGEDLGPLNVK

ELQQLERQLEVALTHLRSRKTQVMLDQIEELRQRERLLHEVNKSLQKKLSETEGRDVITGIEQ

TSNTNTGTNGPWDSSITNTAYALSHPQQDSNSSLHHVDCEPTLQIGYQPVAPESIVPPHQPPH

NQTPNQYMQGWWV

Figure 14

GGCCCGGGCTGGTATTTGGTGTAAGTTTTAAAGGAAGCTGGAAGATTTCAAATCAGTCCT

AATCCTTTAAAAAcTCAGCTTTTGCAAACATGTAACAATCTATGAGTATTATTATGCGTTAT

GGATGTGGCATTTCCACTGCCTTTTAAGTACTTCGGTTTGTAAGTAAAACTAAAACAGAAA

ATTTCTCTTCTAGTATTCTTAAACCGAGTGGAGTTGAGCTGAAGTGAGAGGAGGTATATA

TATATATGTATATTTATTTAGAGTCCAAAGAATCGAGGCGAAAGGCATGTGATGGACGAA

AGCAACCTTAATCTCTCTGCAAATTGTCCCTCCAAATTCCAACTAAAATCAATCCGGACCC

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AATGTATGGGCATGTGGCGCTCATCAATGATGATAGATGGGCATGGGGCCCCACATGAT

GTGGACAGTGGCAACAATAGTACACTAGTTGTAAATGGGTAACCTGTAGTTGGCTTGGTT

TACTTTATATGGAATGAAAGAGGTACGAAACAAAGTATATCAAACGTTGACTCAATGAGT

ACGCCGATCGACGCGTGTTTTTAATTTTTTAATGTTCCAAGGCCGGGCAGGCCCACTTCA

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AGAACAGCAGGAGGAAGCGAAAATTTATTAATAGTAACCAGAGAATAGCAGCGGGTGAA

GAAGCAGAGGGATCTTGCAATGGGGCGGGGTCGGGTTCAGCTGAGGCGAATAGAAAAC

AAAATAAATCGACAAGTCACGTTTTCGAAGCGCCGGAACGGACTGCTG

Figure 15

GAAATTCGGCACGAGGGTAGAGAGATCCCTGGGCCTAGAAGTGTTTTCGGAGGAGCGC

ACTGCATTCTACTTCGGAAAAAAATATGGATGCAGAGCACTTTCCTGTAGGTTTCTTTAGG

TGGGATCAGAGACCAGCACCAGTTGTAGCGGCAGCAGCAGCACCAACAACAACTGTCTT

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GGTCCAAGGAGCCTGGAGAGGATGGGGAGGACAgGCCTAgAgAGCATCCTTTCATTGTC

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GAACGAAaAGCTTAAGATttGGTATGTTCCCACAAAAcTTAGACAACTGTGTCATATGGAGA

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GTGTCACATAGAGAGAAGCAAAAAGTGCCAATAGTTTCATTGAGATTAATGTGTGTAATT

TGAGCTGCTCAGCTTCAAGTGTAGCTCCGTGATTATTGGGCATTTGTGTTCTCATtGTGAC

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ASCKDSTLILQNSNQAQFCGSGLIGVPEHSSESDERKADTNKQKRRRSKEPGEDGEDRPREH

PFIVTEPGELARGKKNGLDYLFDLYEQCGKFLLEVQRIAKEKGEKCPTKVTNQVFRHAKHN

GAVYINKPKMRHYVHCYALHCLDSEQSNHLRRLYKERGVNVGAWRQACYYPLVAIARENN

WDIEGIFNRNEKLKIWYVPTKLRQLCHMERSKECQ

Figure 17

GCCGCACTAGTGATTCGTCCATGGTCCTTGTTAAAGACAGTTGTTGTTGGTGCTGCTGCT

GCCGCTACAACTGGTGCTGGTCTCTGATCCCACCTAAAGAAACCTACAGGAAAGTGCTCT

GCATCCATATTTTTTTCCGAAGTAGAATGCAGTGCGCTCCTACACCAAACAAAAACCACTC

ATAGAATTAGCACATGGGTGATAAATTCTGAgATGCTTTTCATTGATTACCGATTGAACTG

CAAgATTTGAAGTTGATGGGTTACAAATTCCTCCAgATATTTAACCAgACCAAgTGGGTTCC

AAGCGGTACTTGAAgAAgCAgAATgTGTACCTCCGAAAACACTTCTAGGCCCAGGGACCTC

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GTGAgTTTAATGCTtCTTAATCTAGTTTACTGGAACACCATTTTCTTCTAATCACATCaCAGT

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GGCACTTGAAAATTGTTTCAAGTACAATGCAATTTTCCCATGGACTACTGGAACTGCCTG

CTAAAACTGTCTTTTCATGGTCTAATGAGAAGTATATGACCAGGCCTCTTCTCCCTCCATT

TTAAGAAAAACAAATTAAATTATTTAAAAAATAAAATTACaATAATAATCTTGACAGATTGA

TTTTGAGTTGAATGTTAGAATAGATGGAGTGCTGCCTAAGATTTGTTGGCCAAGAAATCC

GCAGCTTATTCCAGAAGTTCACTCATTACAATGTTTTATCTAAGAAATCCTCAACTTATTCC

AAAATGTAACTTATTACAATACCAGCCCGGGCCGTCGACCACGCGTGCCCTATAGTATCC

CGCGGCCATGGCGGCCGG

Figure 18

GGCACGAGCTCGTGCCGAATTCGGCACGAGCGAGTTTTTTTTTTATGGTATTGGAGAAT
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GGAGGGTGGAGAATGAgTATGCCAAAGCTCTGTGATGTTTGTCaGGTATCGAGCTCTGTA
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GTAGCAAGgCTTCGTTGTGTCATGAAAGAgTTTGGGTTTGTGAAGTATGTGAGCAGGCCC
CAGCTGTGGTTACATGCAAGGCAGATGCAGCAGCTTTATGTGTAGCCTGTGATACTGATA
TTCATTCTGCCAATCCTCTTGCAAGTAGGCATGAAAGAGCACCTGTGATTCCATTTTATGA
GTGCCCTAATATGCCCACTAATAATACAGTTACACATGCCAATAATGATAACTTGGACTGC
AATGTTTTGCTGAATGAAGATgGTGGTGGGGATGaTCcTCTGAAACATGATTATGTTGATG
ATGACTATGGTGATTATGATGATGATGAGAATGATCAGAACAATTTGTTGAACAATCaGG
AAgATAATAATGATGCTGAAATATGTTGTGCAgAAgAGgCTGCAACAGCATCATGGTTGAT
TCCTGAGGCCAACAGAAATAATTTGACAATTATCAATGGAGGTAACTCAGAAGGAGAAG
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AAGATGTGGATAATTACGCCGATCTGGAGTACTTGGGAACAACTACTATTACAACGCCAA
TAAACCCCACTGCCAATATGGGGGCAGATAGTATGGTTCCTGTTCACACTCCTGAAGTTA
TTGAGCATTCTTCTACAAAAGTTTCTATTGATACAGCTGGGTCAATGGATGTGGATGCAG
CATCCAAGTGCAATCACgTTTACAGAACTACATCTCTCAATCACtGTGTCTCTTCCTCCCCC
ATAGATGTtGGAATTGTACCTGACAGCAACATTACATCTGATATTTCAACACCTTACCATG
ACCCAAGAGGAGTATTCGAGATTCCTCCTCGGGTTGTTCATCCTGGAGGCCAAGGTGAG
GTCATGGGAAgAGAAgCAAGAGTTCTCAGATACAGAGAAAAAAGAAAGAACAgAAGGTTT
GAGAAgACAATACGATATGCTTCTAGAAAAGCCTATGCAGAGACTCGGCCCAGGATAAAA
GGCCGATTTGCCAAGAGAACAGAGGTAgAAGTGGAACAGATATACTCATcTTCTTTGCTTC
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CTTCAATTTATTCATGCCTATGAGATTGGGCTGGTATGTaAAACTCATGGCAGGTCTATTCa
CATTTGGGTTGTTCGCATACTCAAAGCCTCCTCCTTACTCATTCTGCCTTGCTCATTGATgT
GGgTCAGACTGCAGTAAGATGAAGTCGGTGTGACTCACACTTTGAAGAACAGCCATACAT
ACTATTAGTGTTTCAAGTCGATCGAAGTTTGTTTATTCAGCTTCCTGCAATCTTCTCTGCTC
ATGAATAAATCTTTTCCCCAGTCTCCTACTAAAAAAAAAAAAAAAAAAAA

Figure 19

20/50

A. non - flowering period
   (in all tissues)

laclq

ROS | toxin — CP op anti-toxin — ROS *laclq* NLS

toxin
anti-toxin
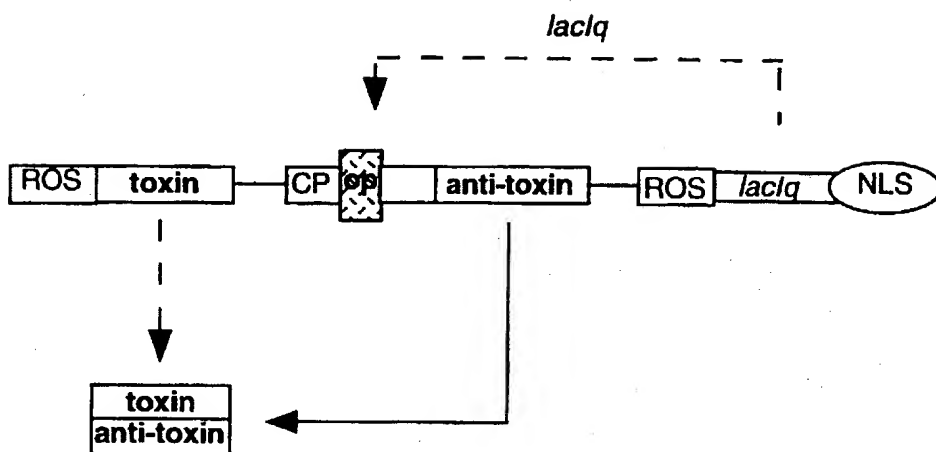
B. flowering period
(in reproductive tissue)

laclq

ROS | toxin — CP op anti-toxin — ROS *laclq* NLS

toxin
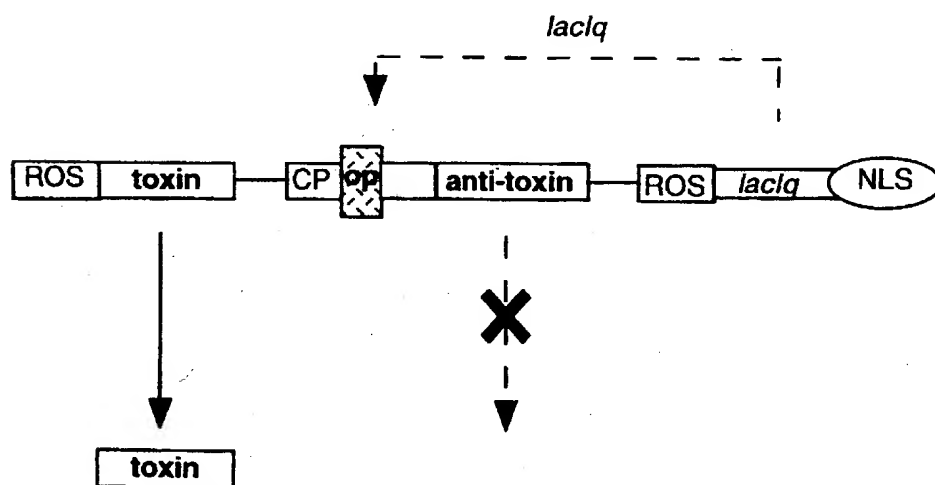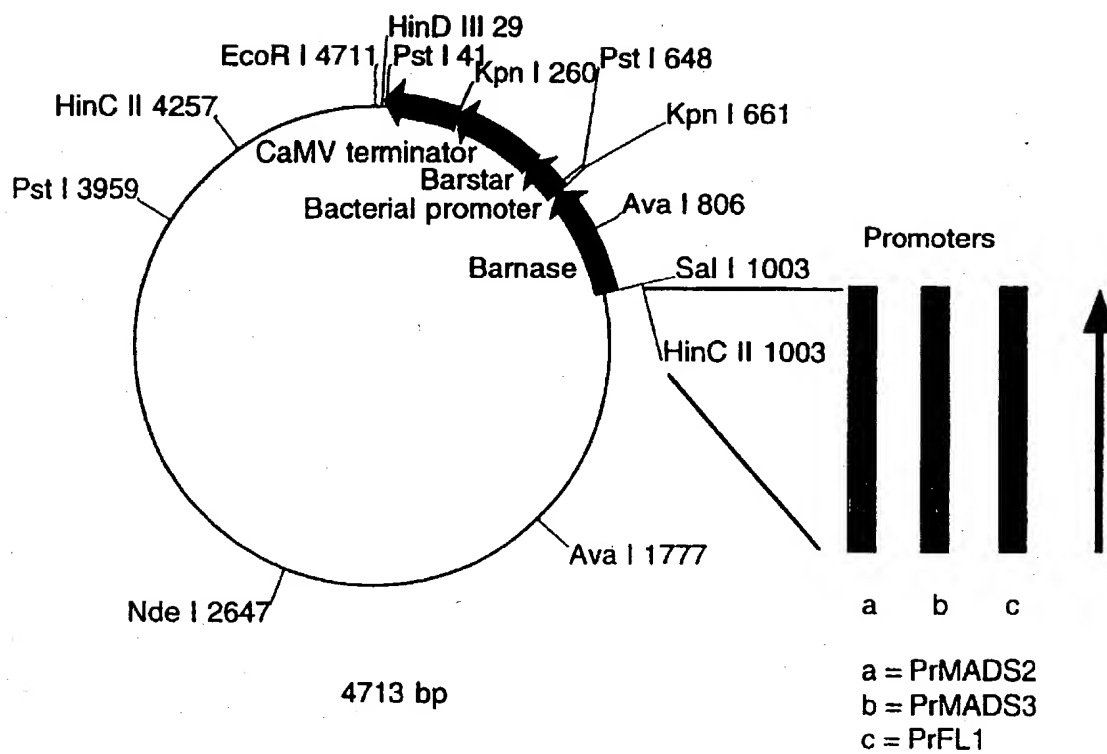
Figure 20

Figure 21

22/50

Figure 21a

6257 bp

Figure 22

24/50

root

seedling

stem

shoot

leaf

mature flower

Figure 24

EGM2   EGM3

receptacle

petal

stamen

carpel

style

mature flower

root

seedling

stem

shoot

leaf

Figure 25

Figure 26

-373
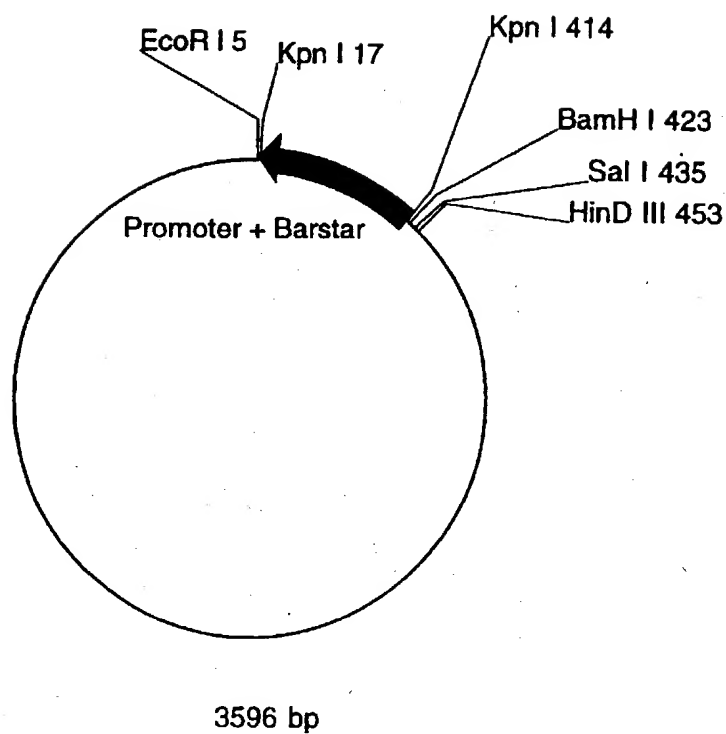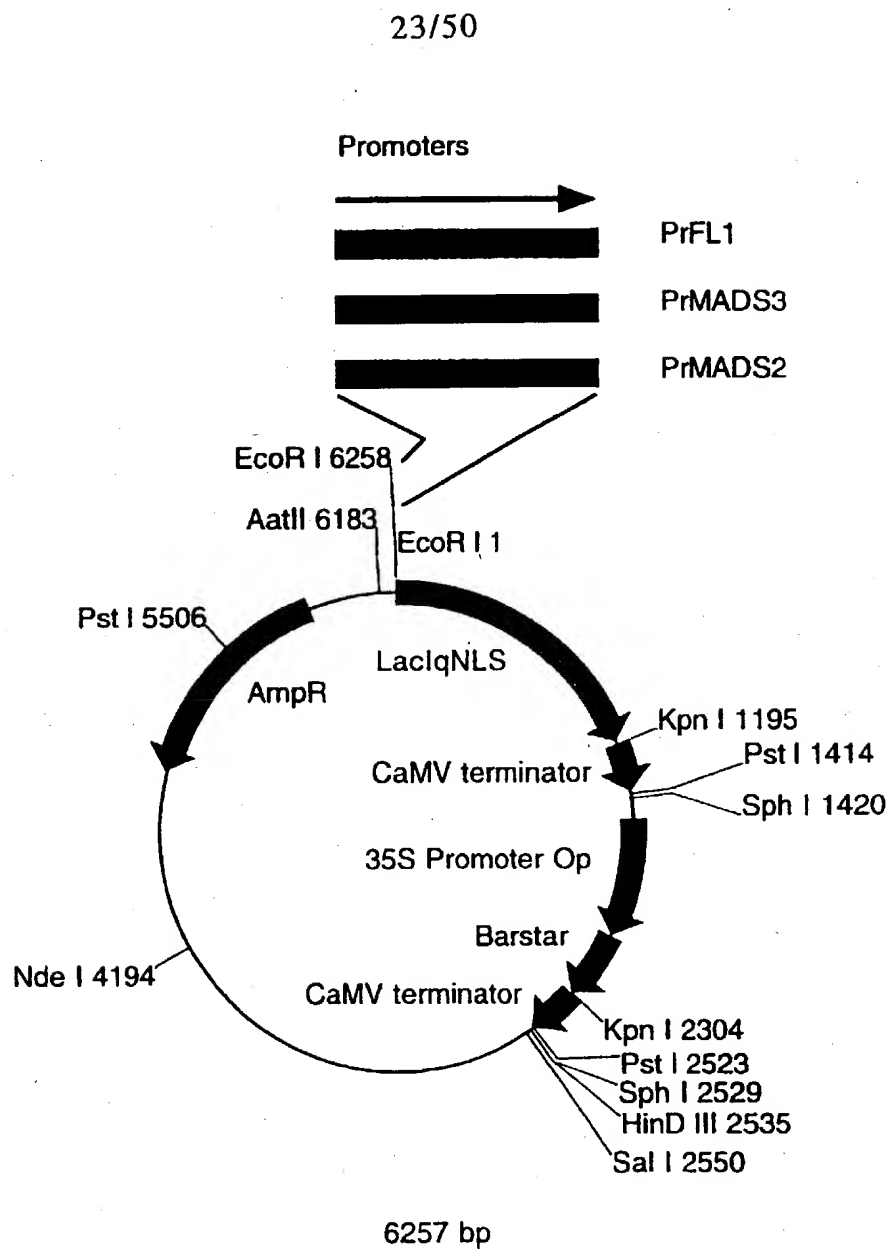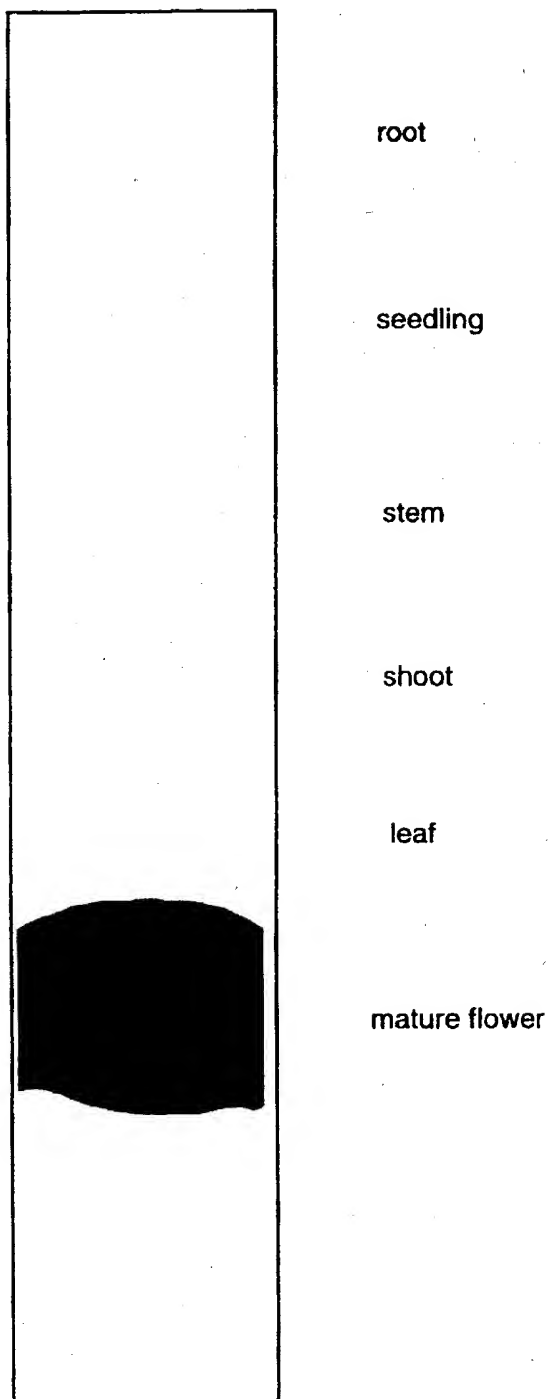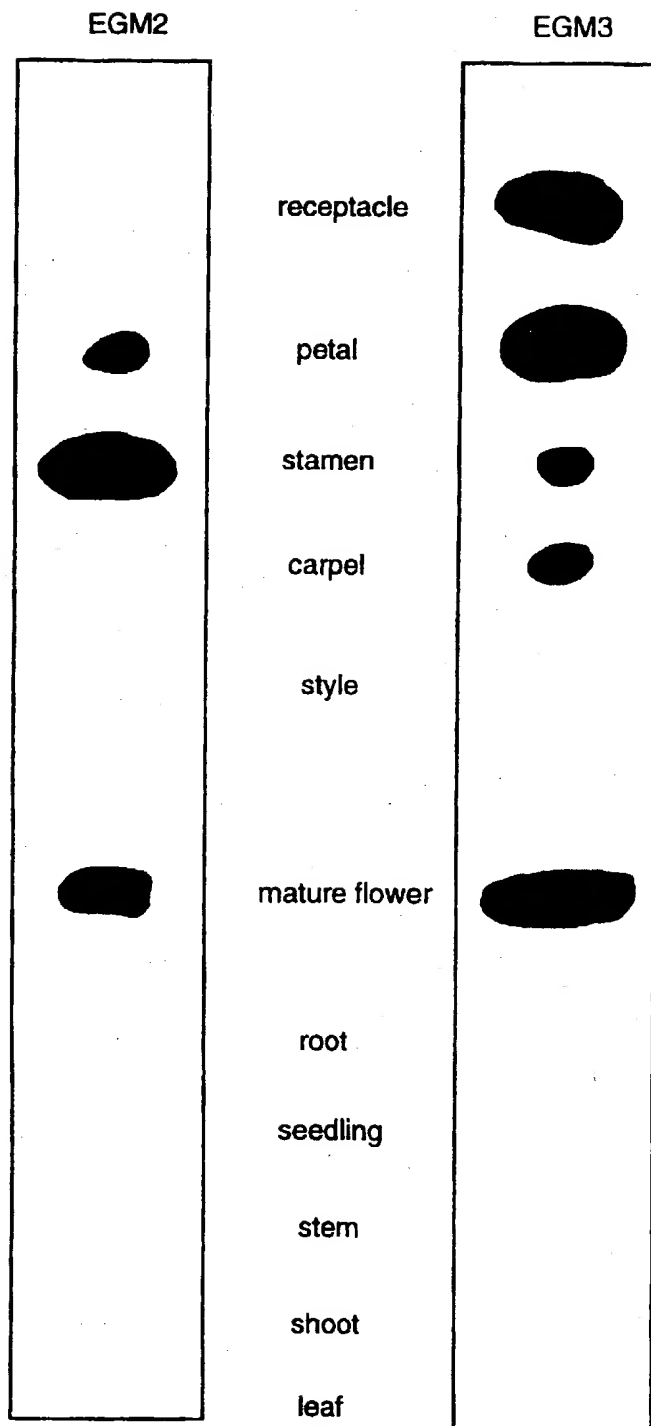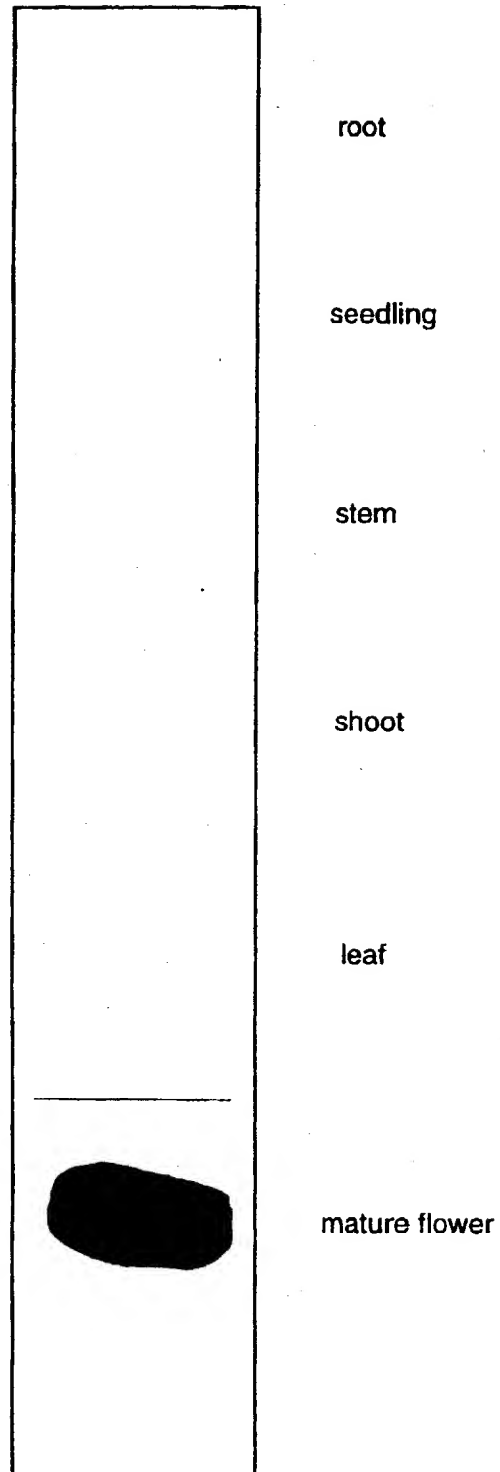
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-293

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TAAAATTCCGCTTCATGACA

-213

AAAAGTACATAGAGGGGCTACTATCTTTGTTGACTTTTACCGTGTAGAGGGTTCCTAAGA

GAGAGGGGGGAGGGAAGAGA

-133

GAGACCATGCATAAGTATGGCTAGACACAAGAAAGGTTGCAGAACCGAGAAGAGATCTC

TTTTGAAGAAGTGAACAAATT

-53

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Figure 28
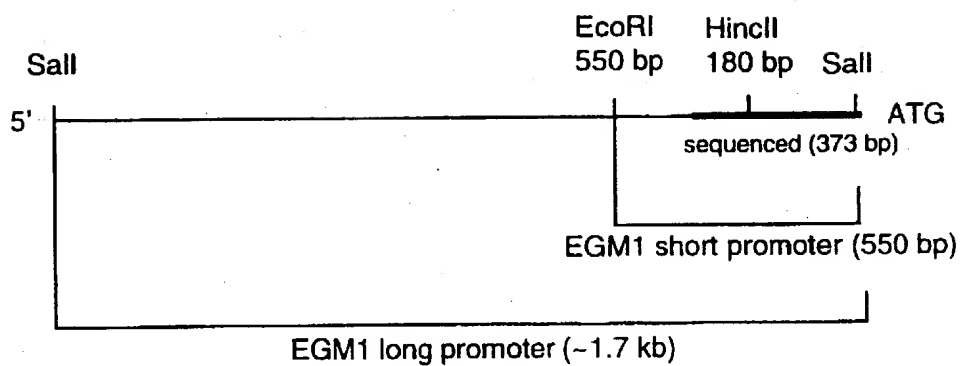
Figure 28A

```
-1191  ATGCTCC     CGACCGCCAT  GGCCGCGGGG  ATTTAATACG  GACTCCACTA
-1145  TAGGGAGCTC  GGAATTCCTT  CCTTTGGAAA  AAGGGGGGAA  AAAATCCGTG
-1095  TCGTTTTTTC  TTTCGTAAAC  AAAAGTCGGA  TGAAATAATA  TGTATTGTTA
-1045  CTATATATAA  AACAAAAGTG  CTCGTGAAAC  ATTGTCAAAT  ACTATAAGGT
 -995  ATAGACAGGT  TTATGACTCA  CAATTACGAA  GGGGCCTAAA  AGCCGAGAGG
 -945  GAAAAGGTAA  TGCTAGGTAT  AGTACATCTA  GACTATTAAT  TGATATAATA
 -895  AGTAAGTGGA  TCATGTTCAA  TTTCAGATAA  AAATTTCTAT  GTCACGTTGG
 -845  TTTAATATTT  GGAATTTAAT  TATTGGATTT  TATTATATTA  TTCTTTGTCA
 -795  TATTAATTAG  ATGGTGGAT   ATTTGCCGAT  TCTCATGAGA  GGAAAATTGA
 -745  TCTTTAATTA  ATTTTCAAAA  TACCCATGGG  CAAGGAAATC  ATAAGGAAAA
 -695  CTCATCTCTT  CCCAACACG   CAACGCCCAC  ATCGTGCTCT  AGGGTTAAAT
 -645  AGAGATGTGA  TCTCCAAATT  TTTTGTCTGG  GAGCAATGCT  AAAGATGCTA
 -595  AAAAGTTTA   GCAAGTAGGA  TGACTAACTT  GAAATAGGGC  AGTCAATAAA
 -545  CCAAGTCTAA  CGATCAATTA  CTACATTACA  CGTAATGAGA  AACATTTCGA
 -495  GAAAATCTCT  CTATTTATTG  TGGTTACATA  AGTAGCCTAC  CTATTTGTAA
 -445  TTATGCAAAT  TAACCTAGGA  CGAGCCATAT  ATGCAGTCGA  CATTCATAAG
 -395  GCTTTATAGT  TTGTGCTTCC  GGAAATAGCT  TAGAAAACCA  CATACACTTT
 -345  TCATCAAGAC  TAATCTGACA  GTAAAAAAG   GAAAAGAAA   AGAACACCAT
 -295  CAAGACTAAT  GTAATGAAAA  GAAAACCCTA  TTACTAATTT  TGAGAGTTTT
 -245  CCTTTGTAGA  CCCATCACGT  CATCCATCTA  AACGATGCA   ATGCACACAA
 -195  CTAATGACAA  GGGAACATGT  TAATATACGA  GCAAACCTAA  CCAGGTTTCC
 -145  CCTTCCTTCA  TTAGAGTATA  ATAACTGTTC  CCTTTTCTAC  CTCCTTTTTG
  -95  TACCCTAGCT  CTCGAGCTCC  TTAAGAAAGT  TTGCTCGGCT  CTTAACCAGG
  -45  ACTAGAAAAG  ATCAGAGAGA  GAAAGAGAGA  AAGAGAGACA  GAGATATGGG
  +5  AAGAGGG
```

Figure 29
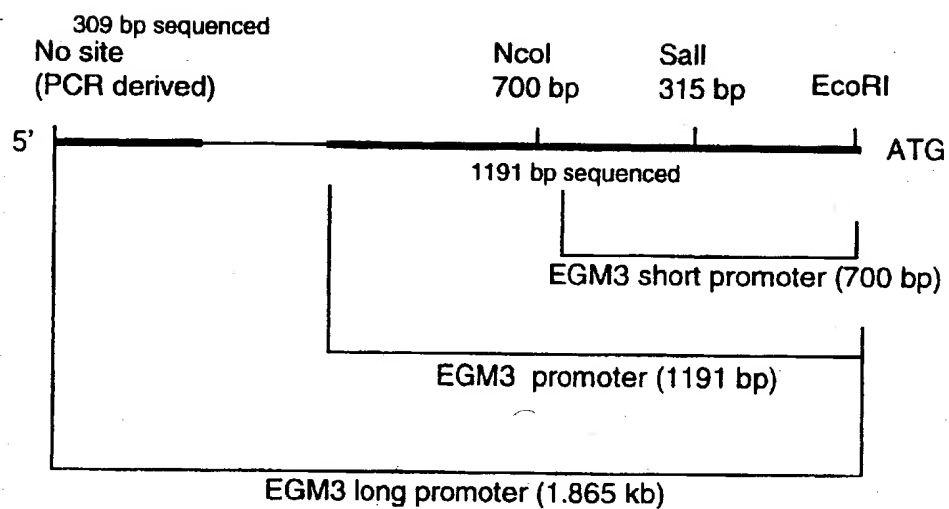
Figure 29A

-1865

TAGCTCTCGAGCTCCTTAAGAAAGTTTGCTCGGCTCTTAACCAGGACTAG

-1815

AAAAGATCAGGGAGAGAAAGAGAGAAAGAGAGACAGGGATATGGGAAGAG

-1765

GGAAAGTATAGCTGAAGAGGATAGAGAACAAAATCAACAGGCAAGTAACA

-1715

TTTGCGAAGAGAAGAAATGGGCTTCTCAAGAAAGCTTATGAGCTCTCTGT

-1665

TCTCTGTGATGCTGAAATCACTAGTGCGGCCGCCTGCAGGTCGACCATAT

-1615

GGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTC

-1565

ACCTAAATA   -1556

Figure 29B

-940

ATAGAGCAGTACACTTTAAGGGAGCACGCACCCCAATTTGATATAAGGTACAAAGTTTTG
TTCCAAGATGTGGGTGACAT

-860

CATTAAATGAGATTTACCATCTTTCAACAGGTTCAAAGGGTACCCTGTACTATAAAGCCCA
CGAAGCTATTTGTATGTTA

-780

AAAAAAAATTCACATAGACTAGGTACGATCGAAAAGCTCATTAAGGAGCTGCCAACGAAA
AGTTCACTTTGTTAACCTCT

-700

CCTCTTATCTTATATTCAATAAGAATATGCACATCGGTTGTGATTTGATGCTCAATTGAACT
TGACATTTGGTTATTGGT

-620

TCTACCCACTTAAACCCTAAGCAAAAAGGCCAGAAATGTGCACACATTATTCATCAAGCC
GGGTGCCACGTGTCCAGTAA

-540

GACAAACTAAATAAGGAGAAAATACTGGTAAGGAAGCCTATGTTGTGTATGTAAGATACC
CAATGATAGTCCGTGGTTTT

-460

GATAAGTTCAAACTTAACATTTGAAAAAGGGACAATATGACGAAGCTGCAGTTAGAACAG
GAGAACACCCCAACCCGAGA

-380

AAATATCCGGTCGTTTAATATCAATAGCTCACGTCAGTGCAGGATGTCTGAAAAYGACCG
ATGGTCACACGCGCGTGACA

-300

TGGAAGAGAAGAACCAATAACCAGAAAGCCCTAATAATGAACAGTAGACTTTAGCATGA
AACCTCTCACCTTTTCTTTTG

-220

TGCCAAGAAAGTCGCAGTTCCCCTTAACCCTAAATGGAATGTTGTGTCTTTACTTCTGTAC
CTCTCCTTTTTTTCAGCCT

-140

CTCTCTCTCTCTCTCTCTCTCTCTCTCAGTCTCAGCTATTCAGCTATTCTGCTCCTCAGCTT
TCATTTGCAAACAAGAGC

-60

AGATAAGGAAGATAATACGAGAGAGAACAAGAGAGAAAGAGAAGGCCCGGAAAAAGAA
AG<u>AT</u>CGGTGCAC

(mutated ATG)

Figure 30

Figure 30A

34/50

PstI   Kb ladder
EGM3
Double Bin

P   K

◄— 5.090

◄— 4.072

◄— 3.054

◄— 2.036

◄— 1.635

◄— 1.018

Weak ►
band   ◄— 0.516 / 0.506
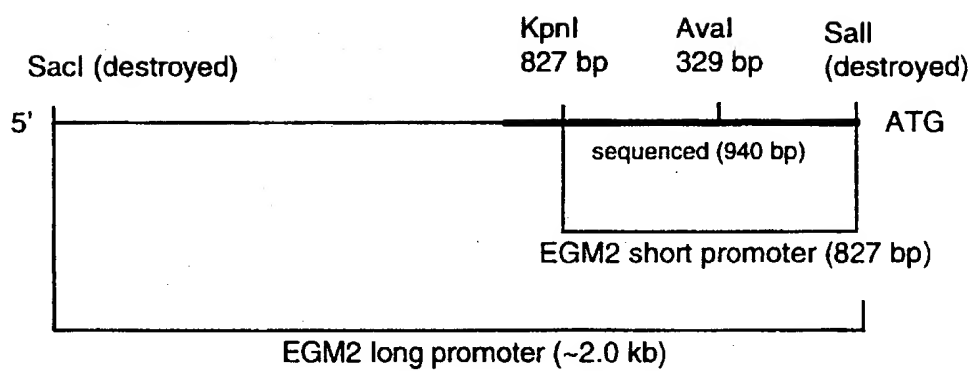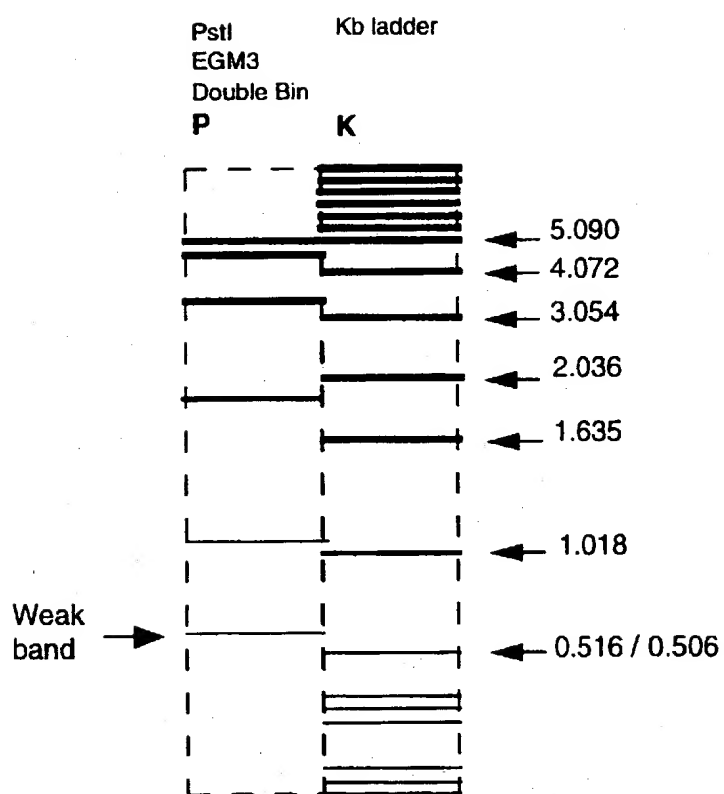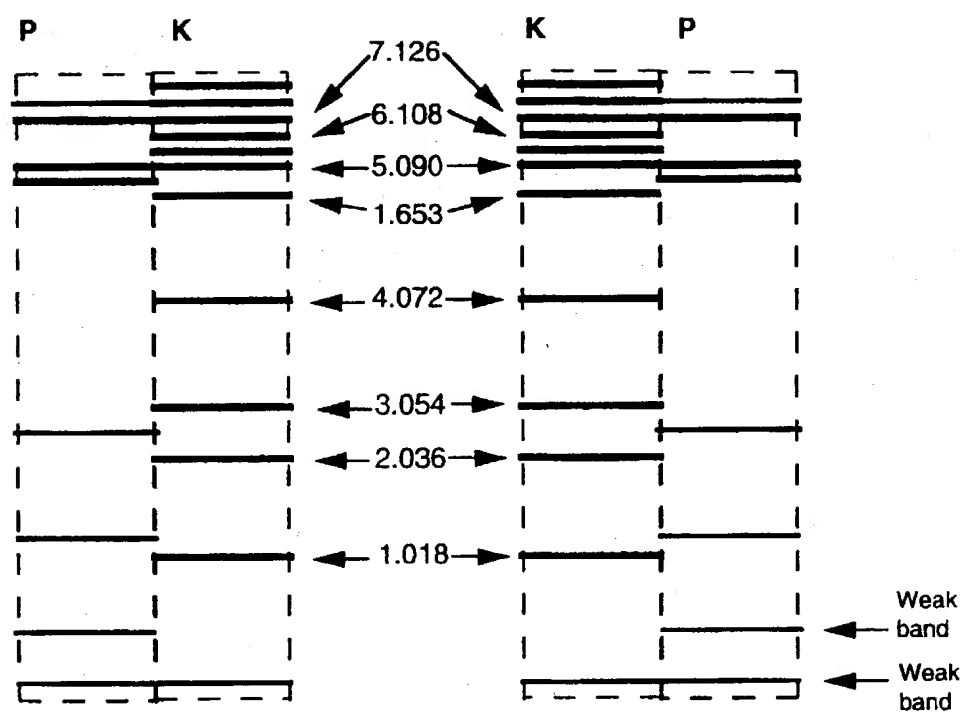
Figure 31

Figure 32

36/50

Figure 33

5809 bp
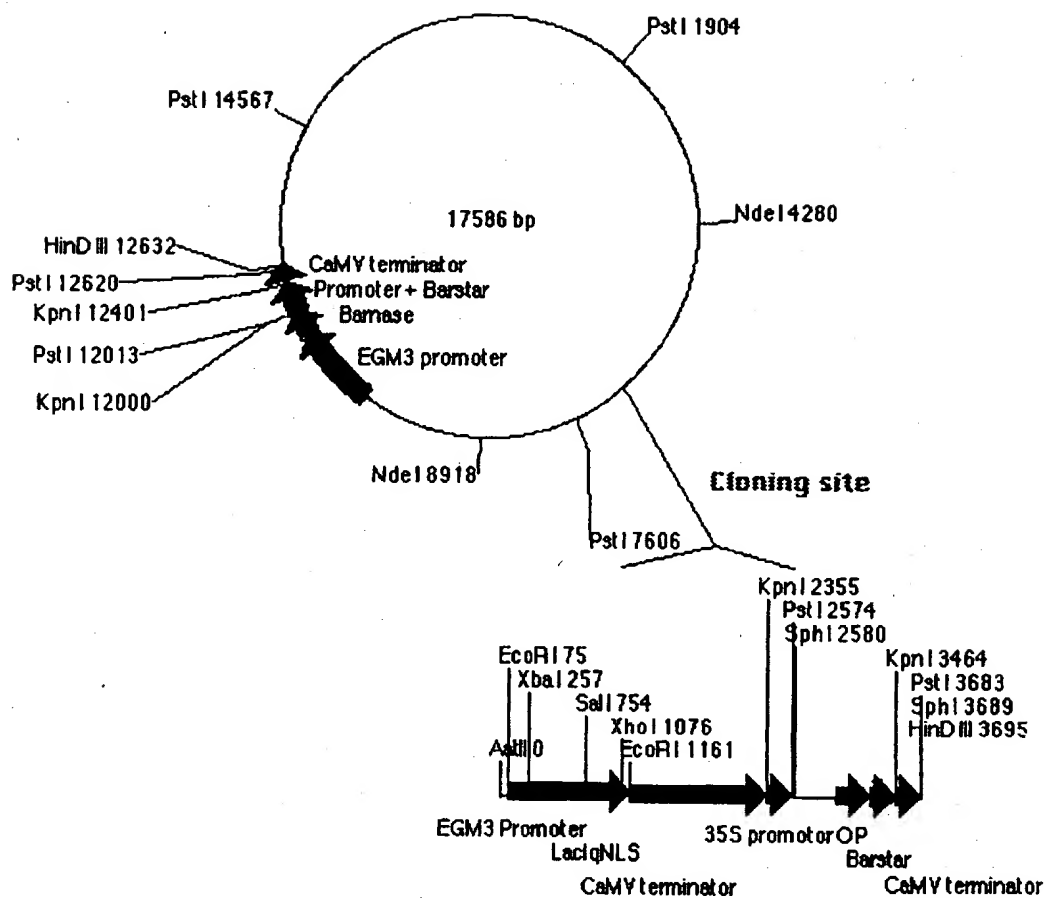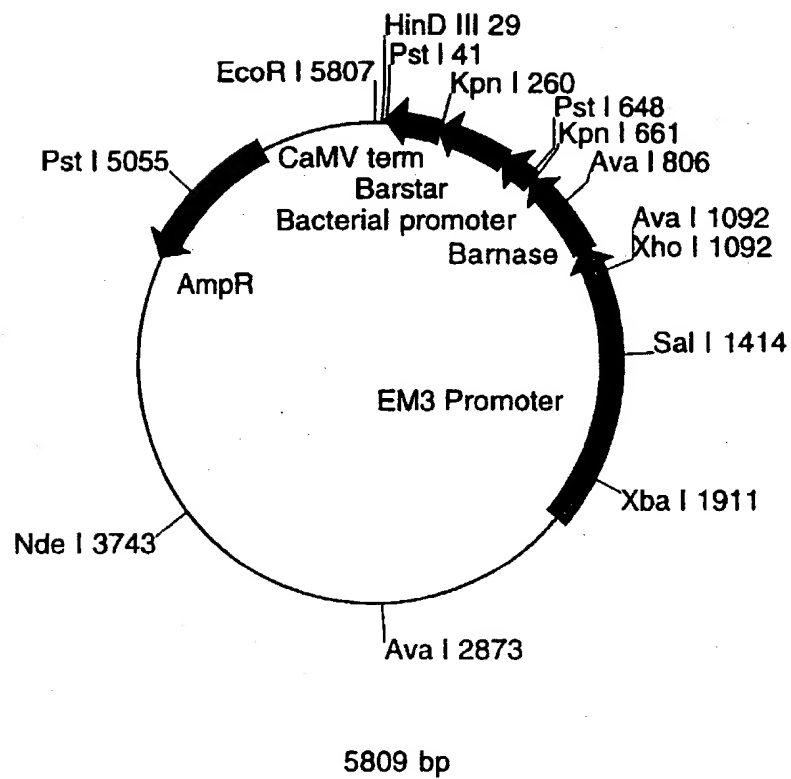
Figure 34

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Figure 35

39/50

4312 bp

Figure 36

40/50

EcoR I 6258

AatII 6183

EcoR I 1

Pst I 5506

LaclqNLS

AmpR

Kpn I 1195

Pst I 1414

CaMV terminator

Sph I 1420

35S Promoter Op

Barstar

Nde I 4194

CaMV terminator

Kpn I 2304

Pst I 2523

Sph I 2529

HinD III 2535

Sal I 2550

6257 bp
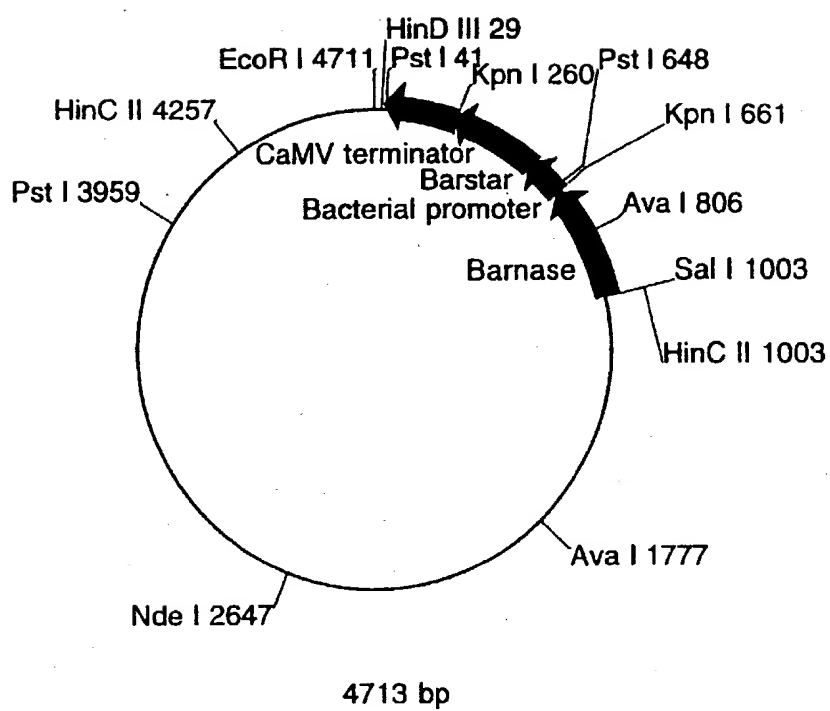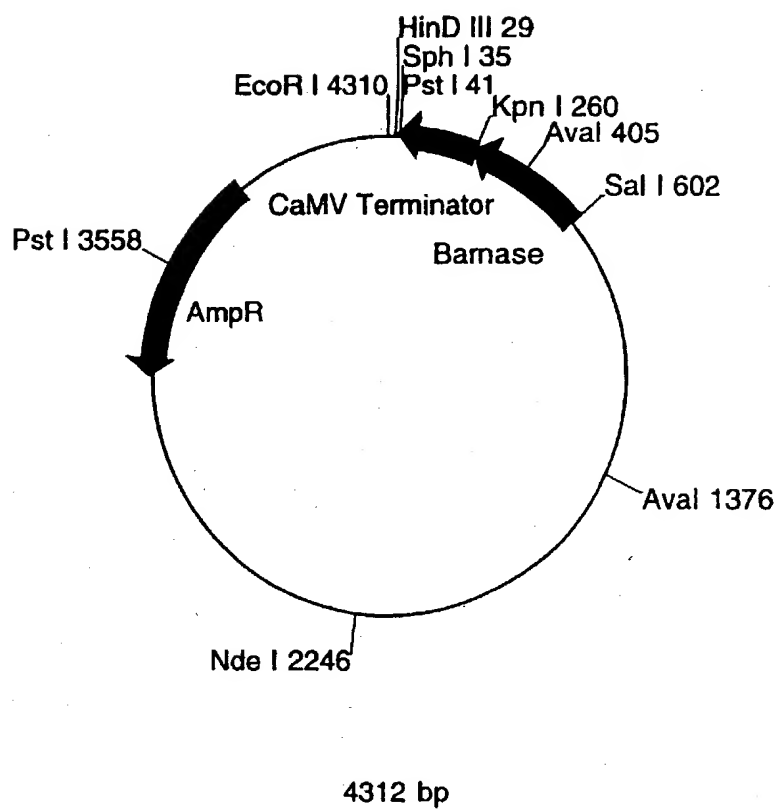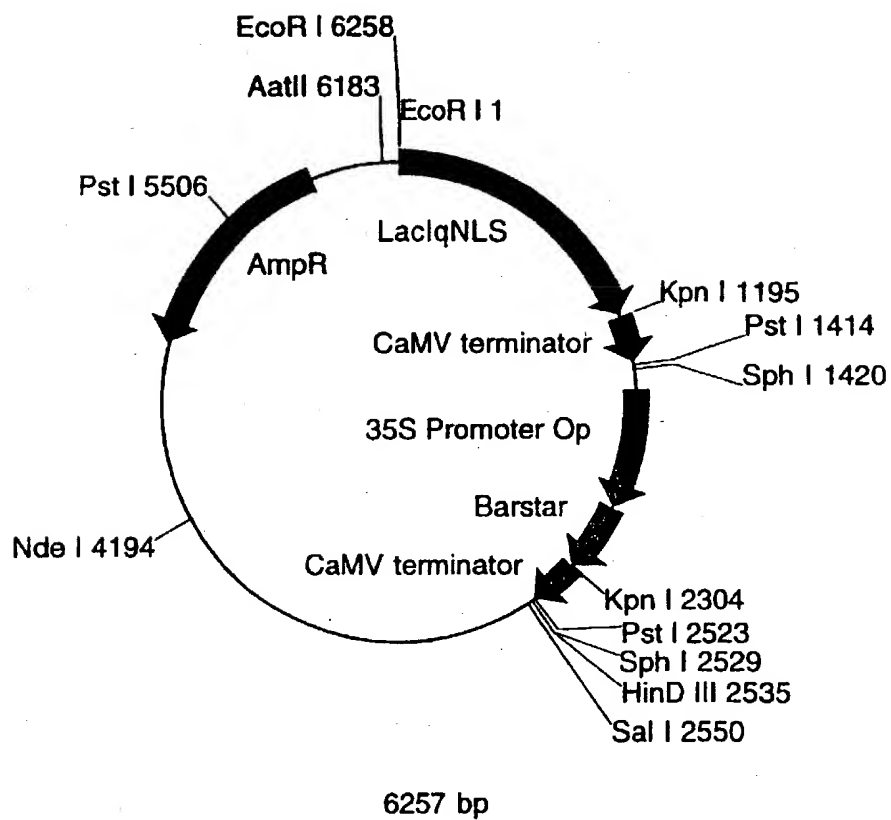
Figure 37

41/50
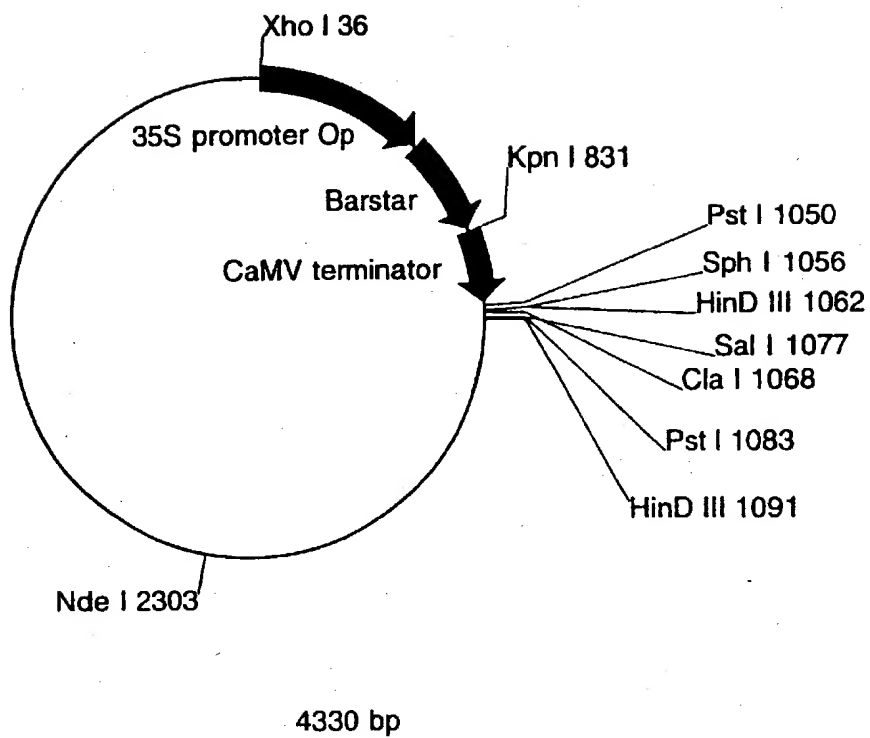
Figure 38

42/50
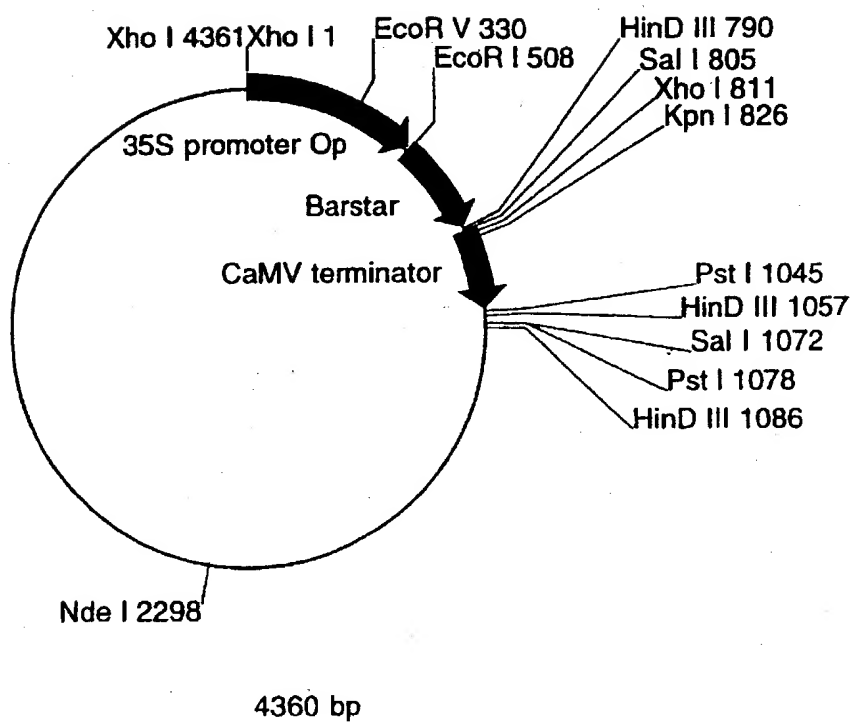
Figure 39

43/50
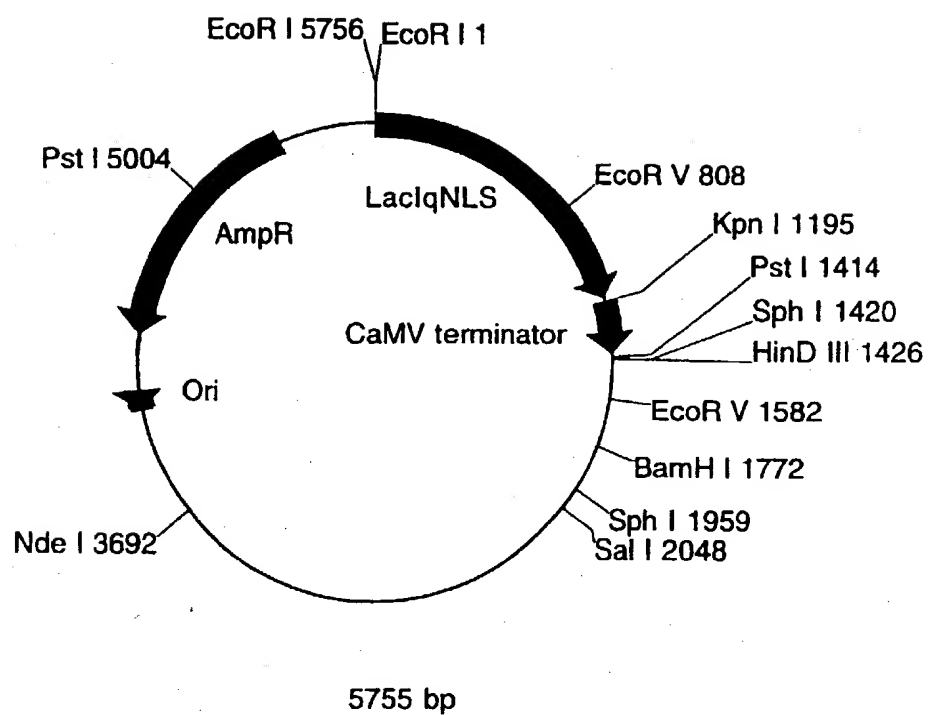
Figure 40

Figure 41

45/50

Figure 42

17586 bp

Figure 43

7343 bp

Figure 44

48/50

5216 bp

Figure 45

49/50

FB.13L30   control
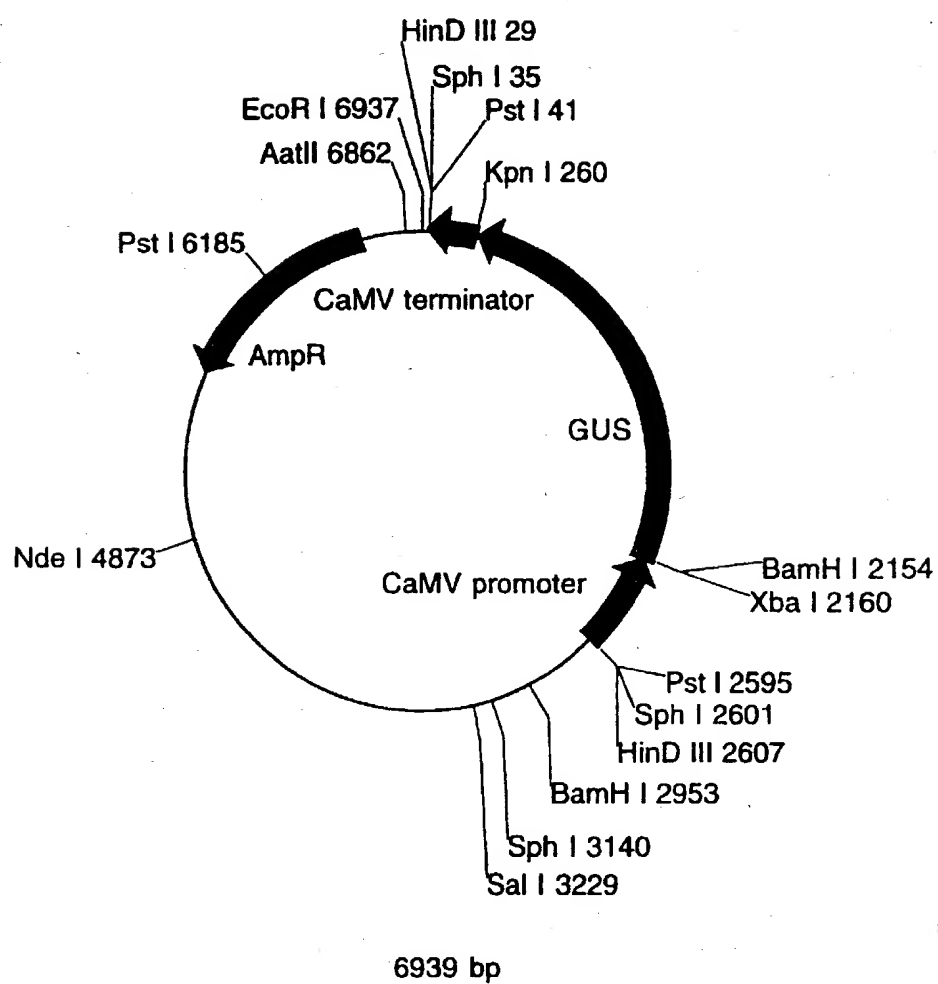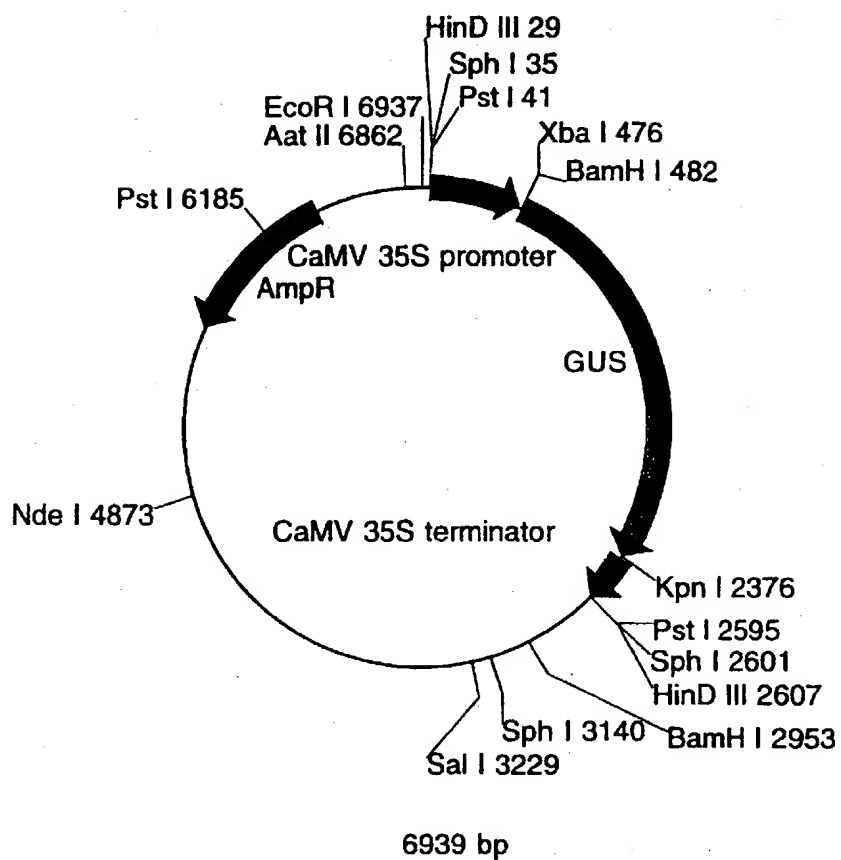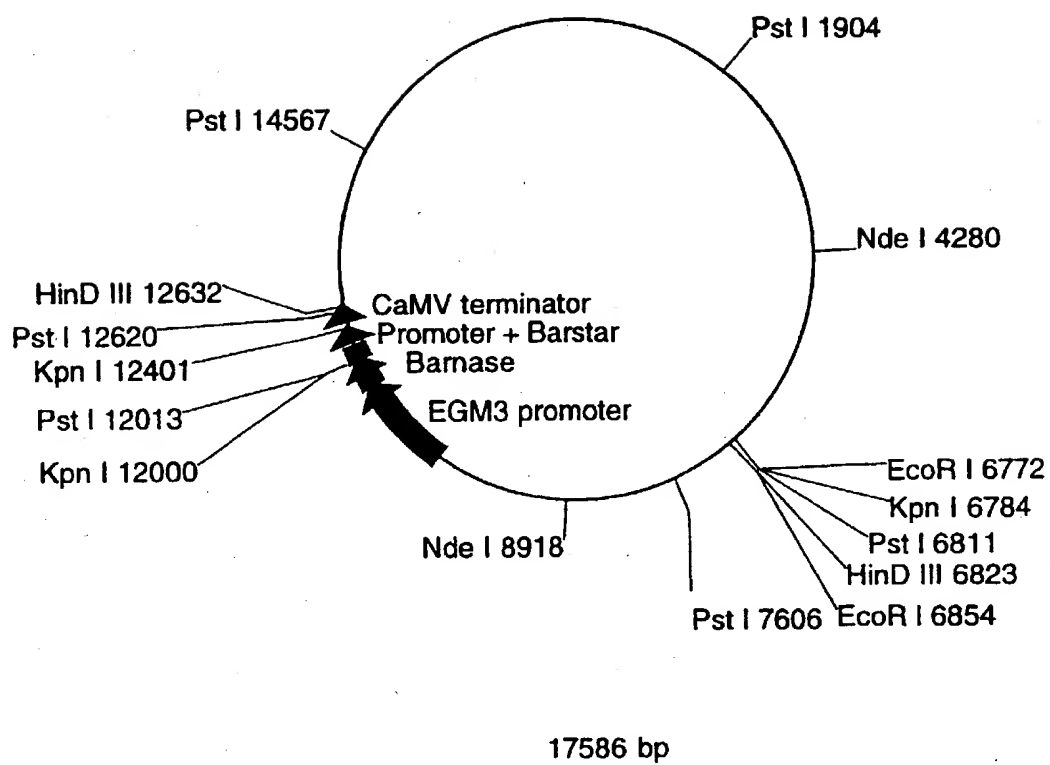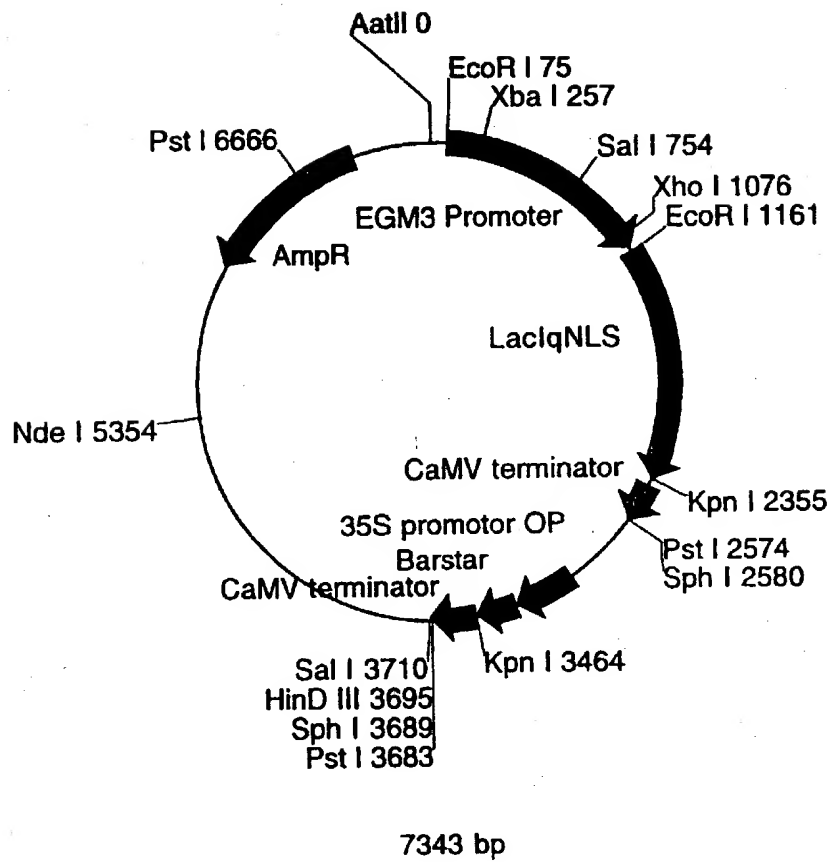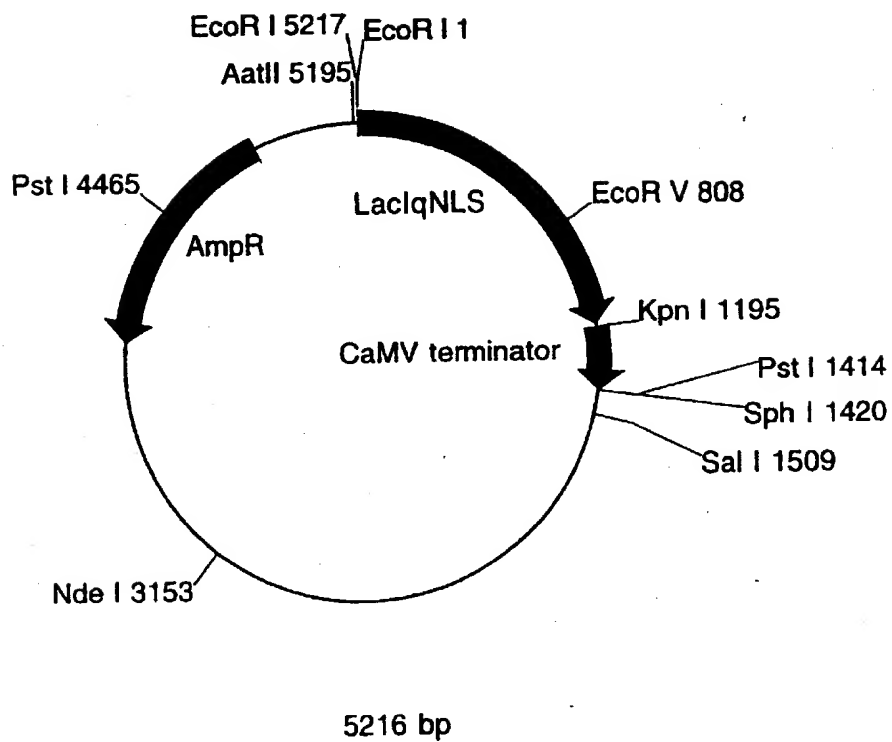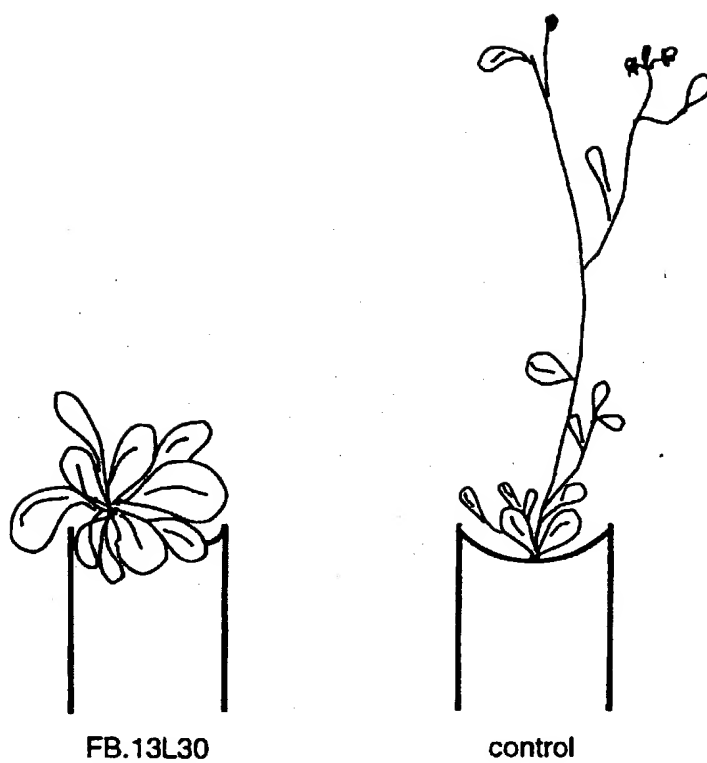
Figure 46

50/50
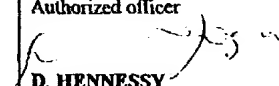
Figure 47

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]:   C12N 15/82, 15/84

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC C12N 15/82, 15/84
CHEMICAL ABSTRACTS

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
(SEE ATTACHED)-ATTACHMENT 1

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X, Y	AU 17132/97 A (FORBIO RESEARCH PTY. LTD.) 21 August 1997. See whole document in particular. See plasmids disclosed.	1 to 29
X, Y	AU 86224/91 B (FB INVESTMENTSPTY.LTD) 15 April 1992. See examples and claims.	1 to 29
X, Y	Strauss, S.H. et al. (1995) Genetic engineering of reproductive sterility in forest trees, *Moleculor Breeding, vol.* 1, 5-26.	

[X]	Further documents are listed in the continuation of Box C	[X]	See patent family annex

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 October 1997	**10 NOV 1997**

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	**D. HENNESSY** Telephone No.: (02) 6283 2255

C (Continuation)	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, Y	WO 94/25613 A (CORNELL RESEARCH FOUNDATION, INC.) 10 November 1994 See whole document	1 to 5
X, Y	WO 94/00582 A (CENTERE FOR PLANT BREEDING AND REPRODUCTION RESEARCH) 6 January 1994. See whole document	1 to 5, 9
P, X, Y	Columbo, L. et al. (1997) downregulation of Ovule-specific MADS BOX genes from Petunia results in maternally controlled defects in seed development, *The Plant Cell*, vol. 9, 703-715. See the plasmids used in particalar.	1 to 29
X, Y	Thorsness, M. K et al. (1993) Genetic ablation of floral cells in arabidopsis, *the plant cell*, vol. 5, 253-261. See the plasmids used in particular.	1 to 5, 9
X, Y	Kandasamy, M.K. et al (1993) Ablation of papillar cell function in brassica flowers results in the loss of stigma receptivity to pollination. *The Plant cell*, vol 5, 263-275-See whole articles	

INTERNATIONAL SEARCH REPORT

Box   **ATTACHMENT 1**

Databases consulted: Derwent 'WPAT', CHEMICAL ABSTRACTS –
                          FILE: CA
                          AGRICOLA

Keywords: PROMOTER, FERTIL:, STERIL:, BARNASE, BARSTAR, REPRODUCT:, OVAR: OR
               STAMENT# OR ANTHER# OR CARPEL # OR CONE# OR FRUIT: (W) BOD:

INTERNATIONAL SEARCH REPORT

Information on patent family members

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report							
AU	17132/97						
AU	86224/91	BR	9106926	EP	550543	CA	2092549
		WO	9205257				
WO	94/25613	AU	68191/94				
WO	94/00582	EP	672155				

END OF ANNEX

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